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Development of methods to assess the fate and effects of *Bacillus thuringiensis* (Bt) Cry1F and Cry3Bb1 proteins

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**Development of methods to assess the fate and effects of *Bacillus thuringiensis* (Bt)
Cry1F and Cry3Bb1 proteins**

by

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A thesis submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of
MASTER OF SCIENCE

Major: Toxicology

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2007

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CHAPTER 1. GENERAL INTRODUCTION AND LITERATURE REVIEW

Thesis Organization

This thesis is organized into four chapters. Chapter One contains a general introduction, which includes research objectives and a literature review on agricultural usage of *Bacillus thuringiensis* (Bt), focusing on biotech crops expressing *B. thuringiensis* genes. In addition, the literature review will detail the terrestrial and aquatic fate of Bt proteins from biotech crops, as well as the non-target effects of both the sporular and transgenic proteins on aquatic organisms. Chapter Two details methods developed to examine the aquatic fate and non-target effects of Bt Cry proteins. In addition, this chapter presents the aquatic aerobic half-life of Bt Cry3Bb1 protein (in leaf, stalk, and root), Bt Cry1Ab protein (in leaf), Bt Cry1F protein (in leaf and stalk), and the dissipation of these proteins in the sediment and overlying water column. Finally, Chapter Two presents the survival and growth effects of MON863 corn root extract containing Bt Cry3Bb1 protein on 10-day old *Chironomus dilutus* larvae. Chapter Three includes new methods developed to examine the fate of Bt Cry proteins in soil microcosms, and compares these methods with previously developed methods. Chapter Three also presents the soil half-life of Bt Cry3Bb1 (in leaf, stalk, and root), the dissipation of Bt Cry3Bb1 in the soil, and the significance of macrodecomposing organisms (isopods, springtails, and earthworms) to the breakdown of MON863 post-harvest crop residue. Chapter Four details improved methodologies for extraction of Bt Cry proteins from soil, which includes the differential recovery of Bt Cry1F protein and Bt Cry3Bb1 protein and the results of a short-term soil monitoring study for Bt Cry1F. In addition, failed attempts at improving Bt protein analysis are presented, including Western Blot verification of intact Bt Cry1F protein and solid phase extraction methods. Chapter Five is a presentation of general conclusions from this research, as well as acknowledgements.

Introduction and Literature Review

Bacillus thuringiensis Agricultural History and Use

Bacillus thuringiensis (Bt) is a rod-shaped, aerobic, gram-positive, spore-forming bacterium that is indigenous to many environments typically colonized by insects, including soil, stored-product dust, and deciduous and coniferous leaves [1,2]. The bacterium was first isolated in 1911 from diseased larvae of the Mediterranean flour moth (spp.). During

sporulation, Bt produces insecticidal crystalline proteins. More than 3000 Bt strains have been isolated (from over 50 countries), which are active against Lepidoptera, Diptera, Coleoptera, or both Lepidoptera and Diptera [3,4]. Since 1961, the U.S. has continually registered pesticides containing spores from a variety of *B. thuringiensis* strains for control of black fly, mosquito, and other aquatic larvae of biting pests [4]. Transgenic crops expressing Bt insecticidal proteins were introduced to the agricultural market in 1996. Since 1996, worldwide usage of Bt crops, specifically Bt corn, has increased dramatically from 1.1 million hectare (ha) Bt corn grown in 1996 to 19.1 million ha in 2006 [5].

Bt Mode of Action

A great majority of the research conducted on the mode of action of Bt Cry proteins has been done on the lepidopteran-active Cry1Ac and Cry1Ab proteins, and has utilized strains of *Heliothis virescens* (tobacco budworm) and *Manduca sexta* (tobacco hornworm) that are Bt resistant. Hence, Bt Cry1Ac/Ab mode of action is the best understood model and is used to generally describe Bt Cry protein mode of action. When the insecticidal crystal proteins (Cry proteins) produced by Bt during sporulation are ingested by a susceptible insect larva, it triggers a series of events that, over the course of several days, ultimately result in mortality. The site of toxicity for both the native and engineered proteins is the insect larva midgut, more specifically the apical membrane of midgut columnar cells [6]. Cry proteins require solubilization and cleavage in the insect midgut to become active. Generally, solubilization requires an alkaline midgut environment (pH 10 or higher); however, there are some exceptions that have been noted. Once solubilized, the crystals release 130- to 140-kDa protoxin peptides. Proteases in the midgut then cleave these peptides to 60- to 65-kDa toxins. The active toxin binds to cadherin-like receptors on the midgut apical membrane, activating intracellular signaling pathways regulated by alkaline phosphatases. The active protein is then cleaved into toxin oligomers, which bind to N-aminopeptidases and other glycosylphosphatidyl-inositol (GPI) anchored proteins in cell membrane lipid rafts. The oligomers concentrate on these lipid rafts, insert into the cell membrane, create a pore, and cause osmotic shock and cell lysis. In addition to pore formation, intracellular signaling pathways are activated that may lead to cellular apoptosis in addition to cell lysis [6,7]. It is the appropriate midgut environment and the presence of Bt protein-binding receptors that confer the high degree of specificity seen in these insecticides. The symptoms of toxicity appear rapidly, and are characterized by a cessation of larval feeding due to paralysis of the midgut, followed by death several days later [4].

Bt Cry1F and Bt Cry3Bb1 Proteins

For this research the lepidopteran-active Bt Cry1F and the coleopteran-active Bt Cry3Bb1 proteins were used as models to develop methods to determine the terrestrial and aquatic dissipation of Bt proteins, as well as to develop methods to determine the effects of Bt proteins on non-target aquatic invertebrates. *Bacillus thuringiensis* Cry1F protein (transformation event TC1507, plasmid insert PHI8999) expressed in transgenic corn is registered under the trade names Herculex® I Insect Protection and Pioneer Brand Seed Corn with Herculex® I. It was registered in 2001 by both Mycogen Seeds (c/o Dow Agrosciences LLC; Indianapolis, IN) and Pioneer Hi-Bred International (Johnston, IA). Bt Cry1F is effective against lepidopteran pests of corn, such as European corn borer (ECB; *Ostrinia nubilalis*), southwestern corn borer (SWCB; *Diatraea grandiosella*), fall armyworm (FAW; *Spodoptera frugiperda*), and black cutworm (*Agrostis ipsilon*). The protein transformation event is registered until 2008 (http://www.epa.gov/pesticides/biopesticides/ingredients/tech_docs/brad_006481.pdf). Genetically modified corn expressing *Bacillus thuringiensis* Cry3Bb1 protein (transformation event MON863, vector insert ZMIR13L) was registered by Monsanto Company (St. Louis, MO) in 2003 under the trade name YieldGard® Rootworm Corn. Bt Cry3Bb1 is active against coleopteran pests of corn, particularly the corn rootworm (CRW), which includes the western corn rootworm (*Diabrotica virgifera virgifera*), northern corn rootworm (*D. barberi*) and Mexican corn rootworm (*D. virgifera zea*, http://www.epa.gov/pesticides/biopesticides/ingredients/tech_docs/cry3bb1/1_%20cry3bb1_exec_summ.pdf). Bt Cry1F and Cry3Bb1 proteins were chosen for this research because they are both economically important (i.e., prevalent) and should be evaluated for potential toxicity in non-target aquatic organisms.

Environmental Fate and Flow of Bt Proteins

Terrestrial Fate of Bt Proteins

The terrestrial fate of Bt proteins is a key parameter governing exposure of non-target organisms in the environment, because the protein will be incorporated into soil with sloughing of root cells, possibly through the release of exudates from roots, and with plant tissue post-harvest. The binding, persistence, and movement of Bt proteins in soil will dictate the potential exposure of non-target species, including aquatic species.

Previous studies have shown that the dissipation of Bt Cry proteins in soil is biphasic. Palm et al. [8] showed a biphasic pattern of degradation for Cry1Ab protein added as crystals

or in transgenic cotton tissue. Cry1Ab concentrations dropped from 225 ng protein/g soil initially to 30 ng/g after seven days in soil. By 30 days, the protein concentrations were still about 10 ng/g in soil. Donegan et al. [9] demonstrated Cry1Ab protein persistence up to 56 days in soil. Later, Sims and Ream [10] evaluated the persistence of Cry2A protein, introduced to soil in transgenic cotton (*Gossypium hirsutum* L.) tissue, by an insect bioassay. They found the time required for 50% dissipation (DT₅₀) to be 15.5 and 31.7 days under laboratory and field conditions, respectively. However, dissipation slowed after the initial decrease in bioactivity, and approximately 20% of the bioactivity remained at 120 days. Sims and Ream [10] projected that approximately 486 g/acre (4 µg/g soil) of Bt protein would be added to soil from a mature transgenic cotton crop, but this remains one of the few quantitative estimates of Bt protein loads to soil, and it was based solely on inputs from above-ground plant biomass. Other studies have shown a much more rapid dissipation rate of Bt proteins in soil. The half-life of purified, bacterially produced Bt Cry1F protein was estimated at less than one day in a laboratory study [11]. In a laboratory soil microcosm study using Pioneer 38W36 leaves expressing Bt Cry1Ab protein, Cry1Ab in soil was not detectable after 14 days [12].

Stotzky and coworkers conducted several studies on the soil fate of Bt proteins, particularly Bt Cry1Ab protein produced by *Bacillus thuringiensis kurstaki* (Btk). They found that Bt proteins bind readily to surface-active particles in soil (e.g., clay minerals and humic acids), do not easily desorb from these particles, and retain their biological activity while adsorbed. This rapid adsorption to surface-active particles reduces the availability of Bt proteins to microbial degradation, thereby increasing their persistence. In one study, purified Cry1Ab protein that was added to nonsterile soil was detected by insect bioassay 234 days after addition, which was the longest time studied [3]. It was observed that Bt corn crop residue broke down at a slower rate than conventional corn biomass. Higher lignin content in that Bt corn transformation event may have caused decreased microbial decomposition, therefore, increasing the persistence of the Bt crop residue itself [13,14]. In addition, Cry1Ab was found to be released from root exudates of corn grown in hydroponic culture, sterile soil, and nonsterile soil [15]. Bt Cry1Ab protein was found in rhizosphere soil of 13 Bt corn hybrids, which represented three transformation events [16]. In another study, Cry1Ab from Bt corn and rice and Cry3A from potato were again found to be released in root exudates of plants grown in both hydroponic culture and nonsterile soil. Surprisingly, Bt Cry1Ac was not released in root exudates from canola, cotton, and tobacco plants. The reason for the differential release of protein from root exudates is unknown [17].

Two recent field studies have examined the soil fate of Cry1Ab protein present in transgenic corn. Zwahlen et al. [18] examined the degradation of Cry1Ab protein from Bt11 corn in representative tillage and no-tillage systems. In an eight-month study, Cry1Ab protein present in corn residue added to litter bags made of curtain material (20 x 20 cm, 5 mm mesh size) and buried in soil was detected by enzyme-linked immunosorbent assay (ELISA) to be 1.5% of the original protein concentration after seven months. In addition, Cry1Ab protein present in Bt11 corn residue on the soil surface was detected by ELISA to be 0.3% of the original protein concentration after 200 days. In each of these representative systems, a biphasic degradation curve was observed. In a study by Baumgarte and Tebbe [19], the fate of Cry1Ab protein present in Mon810 corn grown at two field sites was studied for two growing seasons. In both bulk and rhizosphere soil there were three to seven times greater concentrations of Cry1Ab protein detected by ELISA in the second growing season when compared to the first. In addition, Cry1Ab protein was detected in both the root and leaf residue present on the soil surface prior to second season planting. These studies suggest an accumulation of Bt proteins from one growing season to the next. Conflicting results were found in another study by Dubelman et al. [20]; the accumulation and persistence of Bt Cry1Ab protein from MON810 and Bt11 corn was examined in plots in five corn-producing states after three consecutive Bt corn growing seasons. Both post-anthesis and post-harvest soil samples (15 per plot) collected from all five Bt field plots had no effect on growth of larvae of the European corn borer. The results from this study, contrary to the study done by Baumgarte and Tebbe, indicate no accumulation or persistence of toxic levels of Bt Cry1Ab after three consecutive growing seasons of Bt corn.

Movement and Transport to Aquatic Systems

Very little research has been presented in the peer-reviewed literature that directly pertains to the potential transport of Bt proteins to aquatic resources. However, there are multiple conceivable routes of movement or transport by which Bt proteins could reach aquatic ecosystems (Figure 1). As discussed previously, Bt proteins are thought to bind strongly to soil components, including clays. Because of this, they are likely to be transported while bound to the particulate matter (soil, decaying plant tissue) with runoff or eroded sediment. Soil loss due to runoff and erosion is highly variable and is controlled by many environmental and cultural factors including soil type, slope, climate, and tillage practices. The amount of soil loss from corn fields in the United States has been documented from 0.24-20.5 tons/acre in one year [21,22,23]. Additionally, Saxena et al. [24] reported that a portion of the Bt protein deposited in soil from purified protein, Bt corn biomass, or

from a growing corn seedling is downwardly mobile and detectable in the leachate from a soil column. The vertical movement of Bt Cry1Ab protein decreased as the clay concentration of the soil increased, indicating that in high-clay soils Bt proteins would be transported to aquatic systems via runoff and erosion. A potentially important path of movement of Bt proteins to aquatic systems is the movement of plant material such as leaf litter, pollen, and crop dust. Currently, regulatory agencies (i.e. US Environmental Protection Agency) consider the addition of pollen to aquatic systems to be the only route by which aquatic invertebrates are exposed to Bt proteins. Transport of crop residue and drift of crop dust are exposure routes that have not been studied, but could be very important to understanding the environmental fate of Bt proteins as the biomass of Bt corn added to surface water by these routes could potentially be greater than the addition of pollen. Following harvest there is approximately 5.4-6.9 tons/acre corn crop residue left on or in the soil [18].

Aquatic Fate of Bt Proteins

Thus far, the only study in the peer-reviewed literature examining the fate of Bt proteins in an aquatic environment was conducted by Douville et al. [25]. In this study, the persistence of Bt Cry1Ab protein in surface waters was determined by adding pure Cry1Ab protein endotoxin to either filtered or unfiltered water at a concentration of 10 ppb. The protein half-life determined by this study was 4.4 days in unfiltered water, with no degradation after 7 days in filtered water. In addition, this study quantified the levels of Cry1Ab protein from transgenic corn found in the Richelieu River in an area located in the vicinity of a Bt cornfield. After pollen shed, Cry1Ab was detected in surface water at 0.2 ppb 29.5 km downstream of the Bt cornfield and at 0.5 ppb in sediment 2m upstream of the cornfield. Two weeks following harvest, Cry1Ab was not detected in surface water, but was detected in sediment at 0.7, 0.9, and 0.6 ppb at 2 m upstream, 2 m downstream, and 82 km downstream of the Bt cornfield. The detection of Bt Cry1Ab upstream from the agricultural field may be due to pollen drift. This study provides preliminary evidence that Cry1Ab protein can be transported from a Bt cornfield to an aquatic system, and suggests a short half-life of Cry1Ab protein in surface water. It has been speculated that Bt proteins may be transported to aquatic systems bound to surface-active particles in soil (i.e. clays), since sediments are preferentially transported soil particles consisting primarily of clays; however this is the first study to provide evidence that Bt proteins bind to sediment.

Effects of Bt Proteins on Non-Target Aquatic Organisms

Hazard identification is an important part of all quantitative ecological risk assessments. Despite their persistence in soil and potential for movement to aquatic systems, the hazard of transgenic Bt proteins to aquatic invertebrates has not been assessed. Although the effects of the bacterial formulations of Bt have been characterized, these data do not directly pertain to transgenic Bt crops, due to differences in exposure routes and proteins. Additionally, there are no bioassay methods developed specifically to assess the significance of exposure of aquatic organisms to proteins from transgenic crops, including Bt crops. The lack of bioassay methods and lack of data concerning the non-target effects of transgenic Bt crops on aquatic organisms are limitations in fully assessing their ecological risk. In an environmental assessment of Cry3Bb1 protein in event MON863 corn, the EPA did consider the input of Cry3Bb1 protein from pollen dissolved in the water column, but did not examine any other potential inputs. They found that the addition of pollen into the water was of little consequence since 100% dissolution resulted in a concentration that was well below previously determined toxic levels (http://www.epa.gov/pesticides/biopesticides/ingredients/tech_docs/cry3bb1/2_c_cry3bb1_environl.pdf).

There are data available in the literature on the non-target effects of the dipteran-active *Bacillus thuringiensis* ssp. *israelensis* (Bti) on aquatic organisms. In a short-term study, using a high dosage of Bti, increased drift (i.e. downstream movement of affected aquatic invertebrates) was seen in larvae of the family Simuliidae for less than one day. Some species within the family Simuliidae (black flies) are considered target organisms for Bti, therefore this result is not surprising. In addition, a slight increase in drift was seen in Blephariceridae, which lasted greater than three days [26]. Studies have suggested that specialist predators, feeding exclusively on black fly larvae, may be negatively impacted through reduced prey populations [27,28]. In one of the few long-term field studies examining the non-target effects of Bti on aquatic organisms, it was indicated that disruption in the food web may cause indirect toxicity in the form of decreased total insect richness [29]. Overall, the results from laboratory and field studies conducted on the non-target effects of Bti on aquatic organisms indicate that larvae from the family Chironomidae are the most susceptible, in fact, Bti has been suggested as a control method for pest-species in the family Chironomidae [28]. In a study done by Lee et al. [30], solubilized Bti endotoxin was bound to two types of clay, and *Culex pipiens* mosquito larvae were exposed to the clay-bound Bti by suspension of the clay-Bti complex in water containing *C. pipiens* larvae. The

clay-bound toxin resulted in significantly higher mortality than the free toxin. This study indicates that binding of Bti endotoxin to clay reduces biodegradation and/or produces a greater ingestion of the toxin by filter-feeding organisms. Several studies have examined the non-target effects of the bacterial formulation of the Lepidopteran-active Bt *ssp. kurstaki* (Btk) on aquatic organisms. No significant negative impacts of Btk were found in these studies [31,32,33,34,35].

Problem Formulation and Research Objectives

Despite high adoption rates of commercial Bt crops, there are few quantitative data concerning their environmental fate (either terrestrial or aquatic), movement into aquatic systems or their potential non-target effects in aquatic environments. Other than a modeled estimation of exposure to receiving waters, the current regulatory framework for biopesticide registration set forth by the US Environmental Protection Agency (EPA) does not include an exposure characterization that considers the potential for transgenic Bt protein movement into aquatic systems. With the exception of purified Bt protein and transgenic pollen exposure to daphnia, the US EPA has no established laboratory methods for evaluating the non-target effects of transgenic Bt proteins on aquatic organisms. In order to assess the possible environmental risk of transgenic Bt proteins, the exposure characterization should include an examination of their potential for movement into aquatic systems, and their fate within those systems. In addition, the effects characterization should include toxicity testing of potentially exposed aquatic organisms. Since few standardized methods of aquatic toxicity testing of transgenic proteins are currently available, it is important to develop laboratory methods that can reliably determine potential toxicity of Bt proteins to likely exposed aquatic invertebrates. The methods developed by this research, along with more complete exposure characterization and hazard identification, will be crucial to future quantitative ecological risk assessments of Bt proteins or other plant-incorporated proteins, e.g., “biopharming” products such as vaccines and enzymes.

The specific objectives of this project are:

1. To develop bioassay methods to determine the non-target effects of transgenic Bt proteins on aquatic invertebrates, using Bt Cry3Bb1 protein as a model;
2. To determine the persistence of transgenic Cry3Bb1 proteins in soil and to determine the potential for movement of Cry3Bb1 protein to aquatic environments;

3. To determine the fate of Cry3Bb1 protein in sediment and the overlying water column in aerobic aquatic microcosm;
4. To determine the fate of Bt Cry3Bb1 protein in a soil microcosm, with and without macrodecomposing organisms;
5. To improve extraction methods for Bt proteins from environmental matrices (e.g., soil, sediment, water, and decomposing crop residue).

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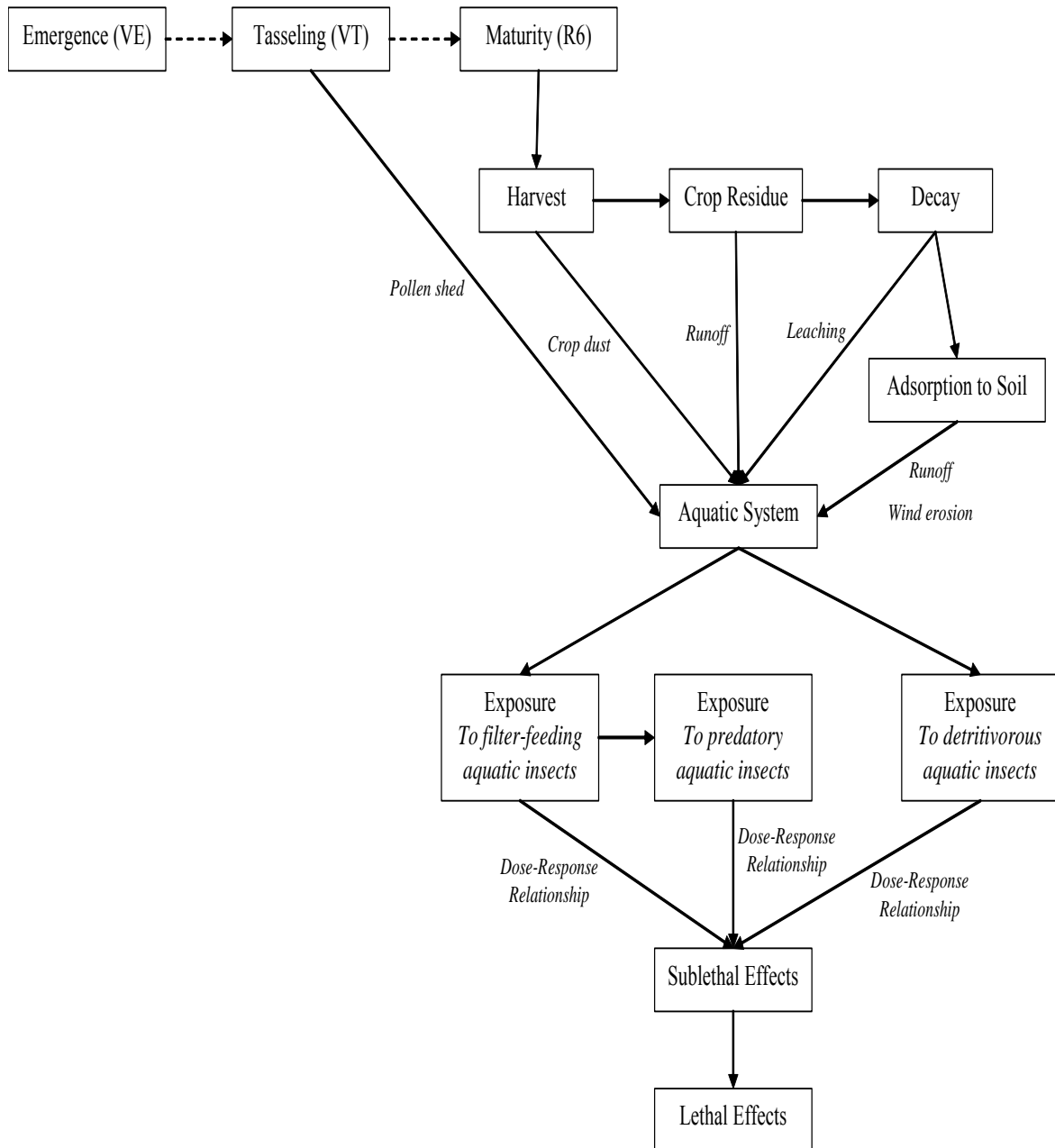


Figure 1. Conceptual model of routes of Bt protein transport to aquatic systems and their potential non-target effects on aquatic organisms.

CHAPTER 2. DEVELOPMENT OF METHODS TO DETERMINE THE AQUATIC FATE AND NON-TARGET EFFECTS OF TRANSGENIC BT PROTEINS ON AQUATIC INVERTEBRATES

Abstract

Biotech crops expressing *Bacillus thuringiensis* (Bt) insecticidal crystalline (Cry) proteins became commercially available in the US in 1996. In 2006, 19 million ha of Bt corn were planted worldwide, which resulted in a significant reduction in the amount of chemical insecticides that are necessary to control agricultural pests. Despite the high adoption rates of this novel insecticide, little is known about the aquatic fate of transgenic Bt proteins and their non-target effects on aquatic invertebrates despite several potential routes of transport to aquatic systems. Methods were developed to investigate the aquatic fate of transgenic Bt proteins and to determine their potential effects on non-target aquatic invertebrates. Laboratory microcosms containing pond water only, as well as pond water and sediment were used to examine the fate of the coleopteran-active Bt Cry3Bb1 protein in decomposing MON 863 corn leaf, stalk, and root. A half-life of less than three days was found for Bt Cry3Bb1 from MON 863 corn residue. No Bt Cry3Bb1 was measured in the pond water or sediment extracts of microcosms containing MON 863 corn. In an acute, static, partial-renewal toxicity test, Bt Cry3Bb1 protein from MON 863 root extracts was fed to *Chironomus dilutus* larvae for ten days. A statistically significant ($p < 0.05$) decrease in *C. dilutus* survival at nominal concentrations of 30 ng/mL was found, however, no effect on growth among the surviving larvae was observed.

Introduction

Bacillus thuringiensis (Bt) is a rod-shaped, aerobic, gram-positive, spore-forming bacterium that is indigenous to many environments typically inhabited by insects [1,2]. During sporulation, Bt produces insecticidal crystalline (Cry) proteins. Since 1961, the U.S. has continually registered pesticides containing spores from variety of Bt strains for control of black fly, mosquito, and other aquatic larvae of biting pests [3]. Crops transgenically expressing Bt insecticidal proteins were introduced to the agricultural market in 1996. Since then, worldwide usage of Bt crops, especially Bt corn, has increased dramatically from 1.1 million ha grown in 1996 to 19 million ha in 2006 [4]. The site of toxicity of Cry proteins is

the insect larvae midgut. Susceptibility requires an alkaline midgut to solubilize and activate the proteins, as well as, the presence of Bt-protein binding receptors (i.e. cadherin-like proteins and GPI-anchored proteins) on the midgut membrane [3,5,6].

The current study used biotech corn expressing *Bacillus thuringiensis* Cry3Bb1 protein (transformation event MON 863, vector insert ZMIR13L), which was registered by Monsanto Company (St. Louis, MO) in 2003 under the trade name YieldGard® Rootworm Corn. Bt Cry3Bb1 is active against coleopteran pests of corn, particularly the corn rootworm (CRW), which includes the western corn rootworm (*Diabrotica virgifera virgifera*), northern corn rootworm (*D. barberi*) and Mexican corn rootworm (*D. virgifera zea*, http://www.epa.gov/pesticides/biopesticides/ingredients/tech_docs/cry3bb1/1_%20cry3bb1_exec_summ.pdf).

There are multiple conceivable routes by which Bt proteins from genetically modified corn could enter aquatic systems from agricultural fields (Figure 1). Currently, the only route that has been examined is the addition of pollen to surface water during tasselling (http://www.epa.gov/pesticides/biopesticides/ingredients/tech_docs/cry3bb1/1_%20cry3bb1_exec_summ.pdf). However, one potentially important path is the direct entry of crop dust during harvest or post-harvest crop residue via runoff into aquatic systems because the transgenic biomass entering surface water through these routes may be much larger than that of pollen. The higher lignin content in Bt corn as compared to conventional varieties may provide increased resistance to microbial decomposition, allowing the crop residue to remain intact on the field for a longer period of time [7, 8]. Bt proteins adsorb rapidly to clay particles and humic acids in soil, do not readily desorb, and the adsorbed proteins are unavailable to be degraded by microbial action [9]. However, adsorbed protein may be more susceptible to chemical degradation. The insecticidal activity of purified Cry1Ab protein from *B. thuringiensis kurstaki* (Btk) was retained for 234 days after addition to nonsterile soil in one study [9]. The strong adsorption of Bt proteins to soil indicates that they are likely to be transported while bound to surface-active particles in sediment eroded by either wind, rain, or snowmelt. Soil loss due to runoff and erosion is highly variable and is controlled by many environmental and cultural factors including soil type, slope, climate, and tillage practices. The amount of soil loss from corn fields in the United States has been documented from 540-46,000 kg/ha in one year [10,11,12]. Saxena et al. [13] reported that a portion of the Bt protein deposited in soil from purified protein, Bt corn biomass, or a growing corn seedling is downwardly mobile and detectable in the leachate from a soil column. The vertical movement of Bt Cry1Ab protein decreased as the clay concentration in the soil

increased, indicating that for high-clay soils Bt proteins could be transported to aquatic systems with runoff and erosion.

Despite the high adoption rates of commercial Bt crops and the possibility for movement into aquatic systems, very little research has been presented in the peer-reviewed literature that directly pertains to the potential transport of Bt proteins to aquatic systems and their fate therein. Douville et al. [14] provides preliminary evidence that Bt Cry1Ab protein can be transported from a Bt corn field to surface water, and that Bt proteins adsorb to sediment in a manner similar to that of soil. Bt Cry1Ab protein was detected following pollen shed in the surface water and sediment of a river adjacent to a Bt corn field. Following harvest, Bt Cry1Ab was not detected in the surface water but was detected in the sediment up to 82 km downstream of the Bt corn.

Thus far, the potential hazard of Bt proteins from biotech crops to aquatic invertebrates has not been assessed, other than the hazard of pollen from Bt corn to daphnia. There are data available in the literature on the non-target effects of the dipteran-active *Bacillus thuringiensis* ssp. *israelensis* (Bti) on aquatic organisms. Overall, the results from laboratory and field studies conducted on the non-target effects of Bti on aquatic organisms indicate that larvae from the family Chironomidae are the most susceptible, in fact, Bti has been suggested as a control method for pest species in the family Chironomidae [15,16,17,18]. In a study done by Lee et al. [19], solubilized Bti endotoxin was bound to two types of clay and exposed to the mosquito larvae *Culex pipiens*. The clay-bound toxin resulted in significantly higher mortality than the free toxin. This study indicates that binding of Bti endotoxin to clay reduces biodegradation and/or produces a greater ingestion of the toxin by filter-feeding organisms. Several studies have examined the non-target effects of the bacterial formulation of the Lepidopteran-active Bt ssp. *kurstaki* (Btk) on aquatic organisms. No significant negative impacts of Btk were found in these studies [20,21,22,23,24].

The sustainability of transgenic crops expressing Bt proteins depends upon a thorough risk assessment, which includes the fate of these novel insecticides in aquatic systems and their potential effects on non-target aquatic organisms. Although the effects of the bacterial formulations of Bt have been characterized, these data do not necessarily pertain directly to transgenic Bt crops due to differences in exposure routes and proteins. Additionally, there are no bioassay methods developed specifically to assess the significance of exposure of aquatic organisms to proteins from transgenic crops, including Bt crops. The lack of bioassay methods and lack of data concerning the aquatic fate and non-target effects of transgenic Bt crops on aquatic organisms are limitations in fully assessing their ecological risk. The

objectives of the current study were to develop laboratory methods that could be used to determine the aerobic aquatic fate of Bt proteins present in biotech crops and to examine their potential effects on non-target aquatic organisms. This study presents data on the acute effects of MON 863 corn root extracts containing Bt Cry3Bb1 protein on larvae of the aquatic midge *Chironomus dilutus*. Bt Cry3Bb1 protein is active against coleopteran pests of corn, in particular corn rootworm larvae. Although *C. dilutus* is a dipteran insect and effects of Bt Cry3Bb1 on this organism would not be expected, it was chosen for this research because it is commonly used in sediment toxicity testing and standard methods for rearing and toxicity testing are available. The aerobic aquatic half-life of Bt Cry3Bb1 protein in MON863 corn leaf, stalk, and root, as well as, the fate of this protein in the sediment and overlying water column are presented in this study.

Materials and Methods

Corn Material

Seeds of event MON 863 corn, transgenic line TP5504-TD, and near-isoline (nearest genetic equivalent) conventional corn line EXP258B were obtained from Monsanto Company (St. Louis, MO). Seeds were planted in 19-L plastic buckets (one seed per bucket) containing Sun Gro® Sunshine LC1 Professional Mix potting soil (Sun Gro Horticulture Distribution, Bellevue, WA, USA). Plants were grown in a greenhouse on a 16 hour light:8 hour dark photoperiod at 27°C (light) and 20°C (dark), and were watered two to three times weekly. Plants were fertilized once, two months after germination, with Miracle-Gro® (The Scotts Company, Marysville, OH, USA) at the label-directed rate. The plants were harvested after maturity was reached (R6 stage) and senescence had begun. Leaves, stalks, and roots of each corn line were frozen at -80°C until the start of the study.

Aquatic Non-Target Effects Study

Test Organisms

Chironomus dilutus egg masses were obtained from existing cultures at the U.S. Geological Survey's Columbia Environmental Research Center (Columbia, MO, USA). *C. dilutus* were reared for three generations at 23°C in a static system according to American Society for Testing and Materials (ASTM) standard methods for culturing and maintaining *C. dilutus* [25].

Study Design

Five nine- to eleven-day old larvae were used per experimental unit. The ASTM standard method for conducting an acute sediment toxicity test with *C. dilutus* [25] was modified for this study; however, the test conditions described in the method were followed closely. The test was an acute, ten-day, static test, with partial renewal on days three and six. The experimental units consisted of 300-mL glass jars, containing 100-mL washed silica sand and 175-mL distilled water. The jars were aerated during the duration of the test at a rate of one bubble per second. Temperature was measured daily in at least one replicate per treatment, and was maintained at $23 \pm 1^\circ\text{C}$. Photoperiod was maintained at 16:8-h light:dark throughout the study. Hardness, alkalinity, and pH were measured on days zero, three, and nine.

Aquatic larvae of the midge *C. dilutus* were exposed to Bt Cry3Bb1 present in the root extracts of MON 863 corn. Only the roots were used because this tissue has the highest expression of Cry3Bb1, and would represent a worst-case scenario of exposure to post-harvest crop residue. A 200-mg sample of MON 863 root was weighed each day of the experiment and extracted by homogenizing in 2.5-mL distilled water in ground glass tissue homogenizer tubes, using a Delta® 9” bench-top drill press (Delta Machinery, Jackson, TN, USA). The extracts were allowed to settle for fifteen minutes, and the supernatant was added to a 4 g/L solution of TetraFin Slurry at the concentrations specified in Table 1.

Larvae were fed daily with TetraFin Slurry (4 g/L) amended with either distilled water (negative control), the orally active, nearly insoluble insect growth regulator diflubenzuron at 20 ng/mL (positive control), or MON 863 corn root extract containing Bt Cry3Bb1 protein at 17 ng/mL (low), 30 ng/mL (medium), or 48 ng/mL (high) TetraFin Slurry. The positive control was used to ensure that the *C. dilutus* larvae were being exposed to each treatment in the test system that was utilized, as diflubenzuron must be ingested for toxicity to occur. There were six replicates for each of the five treatment groups, and five *C. dilutus* larvae per replicate. On day nine, surviving larvae in each treatment were counted and weighed to determine survival and growth during the study. *C. dilutus* weight was determined using dry weight (mg), by drying surviving larvae at 60°C for 24 hours.

The concentration of Bt Cry3Bb1 in MON 863 root extracts to which the *C. dilutus* larvae were exposed (i.e., measured concentration) was determined by spiking TetraFin Slurry to achieve nominal Bt Cry3Bb1 concentrations of 0 (negative control), 17 (low), 30 (medium), and 48 ng/mL (high) in food. The spiked food was centrifuged at $3000 \times g$ to separate the total suspended solids (TSS) from the solution. The concentration of Bt Cry3Bb1 that was unadsorbed (i.e., in solution) was measured using enzyme-linked immunosorbent assay (ELISA). The concentration of Bt Cry3Bb1 that was adsorbed (i.e., in

TSS) was measured using ELISA after extracting the TSS three times with 1-mL of a high-salt buffer at pH 10.5. It was assumed that the *C. dilutus* larvae would ingest only the Bt Cry3Bb1 that was adsorbed to the TSS, as these organisms are filter feeders. Therefore, the measured concentration of Bt Cry3Bb1 in each treatment was defined as the portion of the total protein added that was adsorbed to the TSS in the spiked TetraFin Slurry.

Bt Cry3Bb1 Quantification

Bt Cry3Bb1 concentration in spiked TetraFin Slurry and MON 863 corn root extracts were analyzed using a Cry3Bb1 ELISA kit (EnviroLogix; Portland, ME), and following the manufacturer's instructions. The qualitative kit was made quantitative with the use of a standard curve. Purified (83% pure as determined by SDS-PAGE) *E. coli*-produced Bt Cry3Bb1.11098 (Q349R) protein, provided by Monsanto Company (St. Louis, MO) was used to generate a standard curve. The standard curve was as follows: 20 ng/mL, 10 ng/mL, 5 ng/mL, 3 ng/mL and 1.5 ng/mL. In addition, a buffer blank, negative control (near-isoline corn extract), and positive control (MON 863 corn extract) was run for each plate analyzed. Absorbance (optical density) of samples analyzed with each kit was read against the standard curve at 450 nm (650 nm reference wavelength) on a THERMOMax microplate reader and quantified with SOFTmax software (Molecular Devices; Sunnyvale, CA). Plates having a standard curve R^2 of less than 0.90, or a variability of greater than a 10% CV value were re-analyzed by adding the samples to a new ELISA plate.

Statistical Analysis

Survival and growth for high, medium, and low Bt Cry3Bb1 treatments, as well as for the positive control treatment, were compared to the negative control treatment using Tukey's Studentized Range Test and Dunnett's t Test with Statistical Analysis System (SAS, v. 9.1). Significant differences were defined as $p < 0.05$.

Aerobic Aquatic Fate Study

Study Design

Pond water and sediment were collected from a pond located in Peterson Pits County Park (Gilbert, IA). The sediment was homogenized, and the sediment and pond water were stored in sterilized containers and maintained at 25°C in the dark until the start of the study one week later. Prior to addition to the microcosms, the pond water was filtered through glass wool and the sediment was sieved using a sieve with 2.86-mm mesh to remove rocks and large detritus. Prior to the start of the study, leaves, stalks, and roots of MON 863 corn and near-isoline corn were cut into 5-7 cm pieces, to represent post-harvest crop residue. A total of 0.3 g (0.2% w/v) corn residue (i.e., 0.1 g each leaf, stalk, and root) was added to each

microcosm. There were three treatments, containing either near-isoline (control) or MON 863 corn residue and 150 mL pond water, or MON 863 corn residue and 150 mL filtered pond water and 30 g sieved sediment. Eight time points were used in this study (Days 0, 0.5, 1, 3, 7, 14, 21, and 30). There were three replicates of each treatment at each time point so that at each time point the designated replicates were completely removed from the study and processed. Microcosms were maintained at $23\pm1^{\circ}\text{C}$ in the dark, and were kept on an orbital shaker at 98 rpm to preserve aerobic conditions in the microcosms. Temperature, pH, hardness, and alkalinity were measured in each microcosm on Days 0, 1, 7, 14, 21, and 30.

Microcosm Processing

At each time point, a 1-mL sample of water was taken from each microcosm. All the corn leaf, stalk, and root from each time point was removed from each microcosm and dried in an incubator at 37°C for 30 minutes. A 20-mg sample of dried corn leaf, stalk, and root from each microcosm was weighed. In the treatments containing sediment, the sediment was homogenized, and a 5-g sample from each microcosm was weighed. The water, leaf, stalk, root, and sediment samples were frozen at -80°C until extraction or homogenization. The water was analyzed directly by enzyme-linked immunosorbent assay (ELISA) without extraction. The dried samples of corn leaf, stalk, and root were homogenized using ground glass tissue homogenizers, with an 8" Delta Bench-Top Drill Press, in 1-mL phosphate-buffered saline with Tween-20® (PBST) for one minute. The homogenate was allowed to settle for 15 minutes and the supernatant was removed. The supernatant was frozen at -80°C until ELISA analysis. The sediment was extracted three times with 10-mL of a high-salt buffer at pH 10.5, described by Palm et al. [26] with an orbital shaker at 300 rpm for one hour. The extract was centrifuged at 3000 rpm for 10 minutes, and the extracts from each microcosm were pooled for ELISA analysis.

Bt Cry3Bb1 Quantification

Bt Cry3Bb1 concentration in MON 863 and isoline corn leaf, stalk, and root, as well as in sediment extracts and water samples were measured using a Cry3Bb1 ELISA kit (EnviroLogix; Portland, ME), and following the previously described procedures.

Statistical Analysis

Half-lives for Bt Cry3Bb1 protein in MON 863 corn leaf, stalk, and root from the water-only treatment and the sediment-added treatment were calculated according to first-order degradation kinetics using JMP software (v. 6.0). The nonlinear model, $f = a \cdot \exp(-b \cdot x)$, was used to fit the data for each tissue type in each treatment. For this model, f is the response variable (percent Cry3Bb1 applied), a determines the asymptotes of the curve, b is the first order rate constant, and x is the time in days. The dissipation curve for each MON 863

tissue type was compared for the water-only and the sediment-added treatments using JMP. Differences between treatments for the dissipation curve of each tissue type were determined by calculating F-values with statistical significance defined at $p < 0.05$ [27].

Results

Aquatic non-target effects study

Water quality parameters remained stable throughout the duration of the study. Temperature was maintained at $23 \pm 1.5^\circ\text{C}$. Hardness, alkalinity, and pH ranged from 10-40 mg/mL CaCO_3 , 40-80 mg/mL CaCO_3 , and 7.3-7.8, respectively.

The average concentration of Bt Cry3Bb1 present in MON 863 root tissue was $1.5 \mu\text{g/g}$, while the average concentration of Bt Cry3Bb1 protein present in MON 863 root extracts was 120 ng/mL. For those treatments to which MON 863 root extract was added, 3-5 % of the total Bt Cry3Bb1 protein present in TetraFin Slurry fed to each replicate was adsorbed to the food particles (TSS), and thus available for the *C. dilutus* larvae to filter from the test chamber water. In addition, 35-43% of the Bt Cry3Bb1 protein present in the spiked TetraFin Slurry was unadsorbed, or in solution. Finally, 54-60% of the Bt Cry3Bb1 protein was unrecovered from the spiked TetraFin Slurry (Figure 2). Table 2 lists the nominal concentrations of Bt Cry3Bb1 protein in the TetraFin Slurry, which is based on the average concentration of Cry3Bb1 in MON 863 root extracts, and the measured concentration of Bt Cry3Bb1 protein adsorbed to the suspended solids in the spiked TetraFin Slurry, which is based on the amount of adsorbed and unrecovered (i.e., unextractable) Cry3Bb1.

There was 0% survival of *C. dilutus* larvae in the positive control (20 ng/mL diflubenzuron) treatment (Figure 3), indicating that larval exposure occurred in the manner that the Bt Cry3Bb1 was delivered. There was a significant decrease in survival in the medium and high Bt Cry3Bb1 treatments, as well as the positive control, when compared to the negative control (Figure 3). Among the surviving larvae, growth was not affected in any treatment when compared to the negative control (Figure 4).

Aerobic Aquatic Fate Study

There was no Bt Cry3Bb1 protein measured in the corn tissue of the near-isoline treatment at any time point. In addition, there was no Bt Cry3Bb1 measured in the water or sediment extracts of the near-isoline, MON 863 water only, or MON 863 sediment-added treatments. Rapid dissipation was observed for Bt Cry3Bb1 protein present in decomposing MON 863 corn leaf, stalk, and root. There was no significant difference in the nonlinear

models for MON 863 corn leaf, stalk, and root present in microcosms with and without sediment. Therefore, the data from both treatments were combined, and were fit to a nonlinear model that was used to calculate half-lives for Bt Cry3Bb1 in MON 863 corn leaf, stalk, and root tissue in both treatments. The longest half-life was observed for Bt Cry3Bb1 present in MON 863 corn leaf at 2.9 days (Figure 5). The half-lives of Bt Cry3Bb1 in MON 863 corn stalk and root were both less than one day, specifically 0.6 day and 0.4 day, respectively (Figures 6 and 7).

Discussion

Useful methods to determine the aerobic aquatic fate and potential non-target effects of transgenic Bt proteins on aquatic invertebrates were developed from these studies. For the aquatic effects study, the use of the nearly insoluble insect growth regulator/insecticide diflubenzuron as a positive control demonstrated that oral exposure occurred in the test system utilized in this study, as this insecticide must be ingested for toxicity to occur. A greater range of Bt Cry3Bb1 concentrations could have been achieved with the use of root tissue harvested at anthesis, rather than at senescence, as this is the time when the crop expresses the greatest concentration of Bt proteins in its tissues. However, root tissue may seldom get displaced in an aquatic system. For this study, conclusions can only be made regarding the effects of MON 863 corn root extract containing Bt Cry3Bb1 protein on *C. dilutus* larvae and more research is needed to confirm whether the effects seen were due to Bt Cry3Bb1 or other compounds in the extract. This preliminary study demonstrated a significant decrease in *C. dilutus* survival exposed to MON 863 corn root extract containing Bt Cry3Bb1 concentrations of 30 ng/mL and above, however, more research is needed on both the environmental fate and effects of transgenic Bt Cry3Bb1 before definitive risk conclusions can be made. Growth of *C. dilutus* larvae was not affected by exposure to MON 863 root extract containing Bt Cry3Bb1 protein in this study.

The short half-life of Bt Cry3Bb1 protein in decomposing MON 863 corn leaf, stalk, and root indicate that aquatic invertebrates will experience only an acute exposure of this protein. Although acute effects on survival were seen in the aquatic effects study, this study did not realistically replicate the exposure pattern that would be seen in an aquatic system. It is likely that aquatic invertebrates will be exposed to acute concentrations of Bt proteins present in crop residue, however, there are multiple times during the year when runoff of corn residue is likely to occur. Therefore, aquatic invertebrate exposure to Bt proteins will likely be short-term but may occur at multiple times throughout a year. More research is needed to

determine whether this “pulsed” exposure pattern will have any effects on *C. dilutus* and other aquatic invertebrates. In the only monitoring study that has been conducted thus far, Bt Cry1Ab protein was found at below 1 ng/mL in the surface water and sediment of a river adjacent to a Bt corn field. The concentrations of Bt Cry3Bb1 used in the current study to determine effects on *C. dilutus* larvae were greater than ten times this concentration. More monitoring studies are needed to determine the concentration and frequency of the occurrence of Bt proteins from transgenic crops in aquatic environments. However, based on this study, Bt Cry3Bb1 protein does not appear to be a risk to aquatic larvae of *C. dilutus* based on the short duration of exposure that these organisms will experience in the field.

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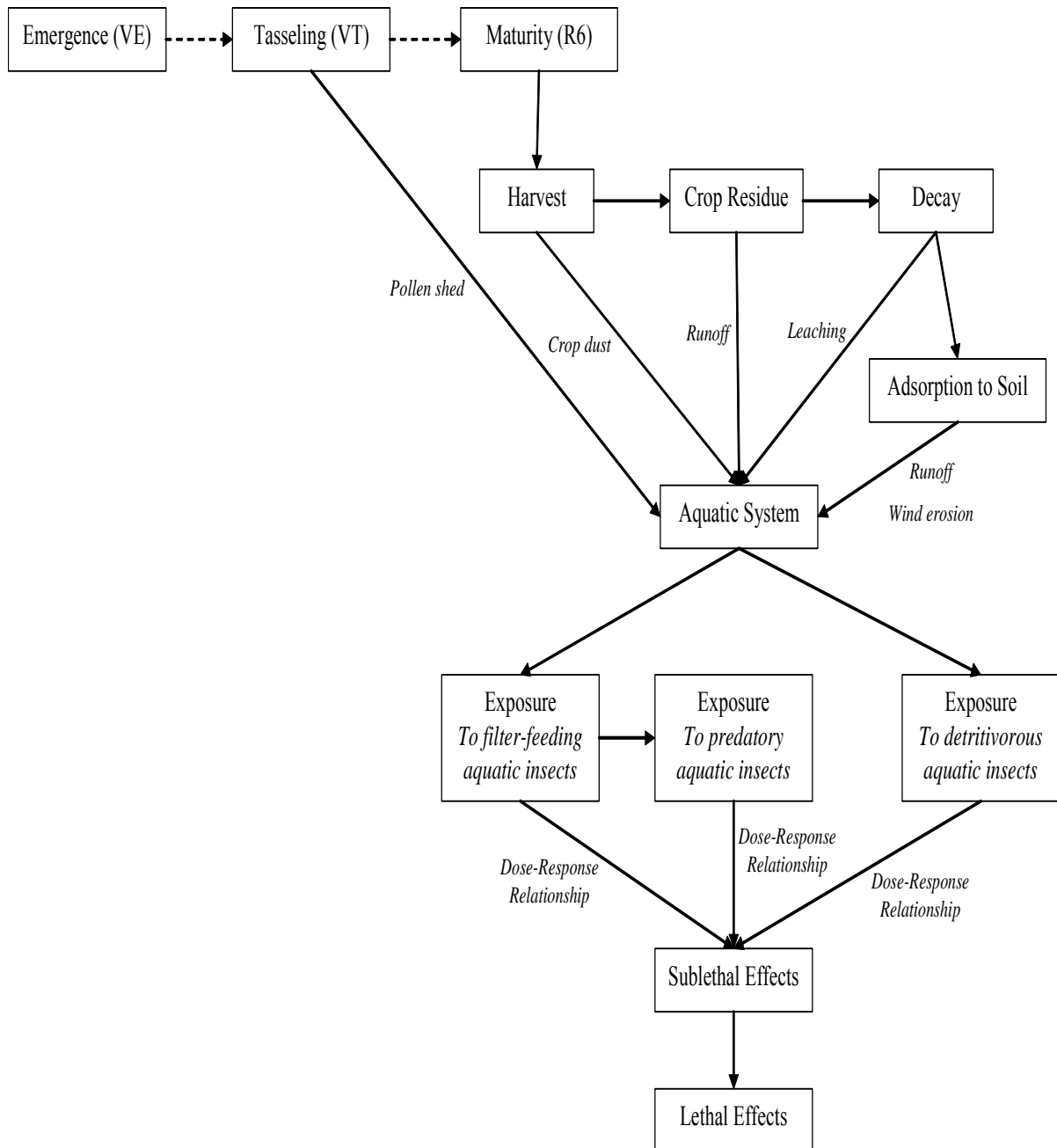


Figure 1. Conceptual model of potential routes of transport of Bt proteins from transgenic corn to aquatic systems and possible effects of Bt proteins on non-target aquatic invertebrates.

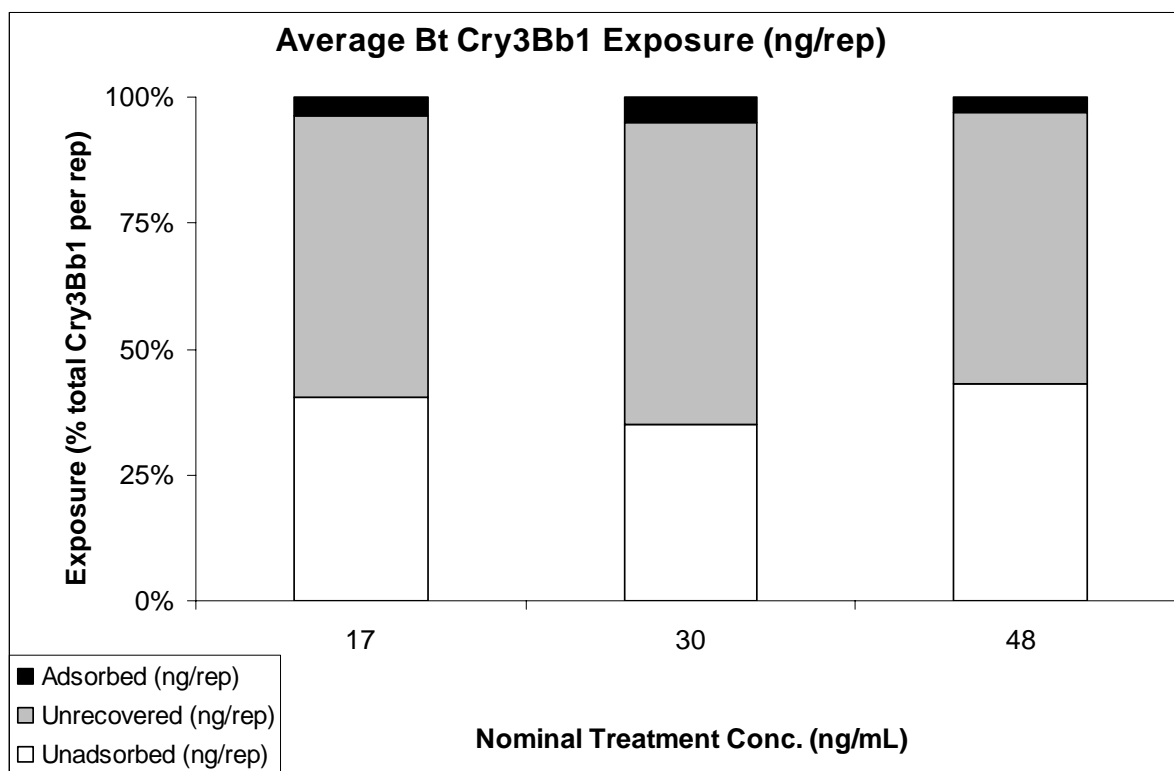


Figure 2. Average percentage Bt Cry3Bb1 protein measured in spiked TetraFin Slurry that was adsorbed to total suspended solids, in solution, and unrecovered in treatments containing 17, 30, and 48 ng/mL Bt Cry3Bb1 protein from MON 863 root extracts. N = 3 replicates per treatment.

Table 1. Nominal and measured Bt Cry3Bb1 protein present in spiked TetraFin Slurry fed to *C. dilutus* larvae. Measured concentrations include the portion of total protein added that was adsorbed to the total suspended solids in spiked diet. N = 3 replicates per treatment. NA = not analyzed.

Treatment	Toxicant	Nominal Conc. in Food (ng/mL)	Measured Adsorbed Conc. in Food (ng/mL)
Negative Control	Distilled Water	0	0
Positive Control	DiFlubenzuron	20	NA
Low Protein	Bt Cry3Bb1	17	10
Medium Protein	Bt Cry3Bb1	30	19
High Protein	Bt Cry3Bb1	48	27

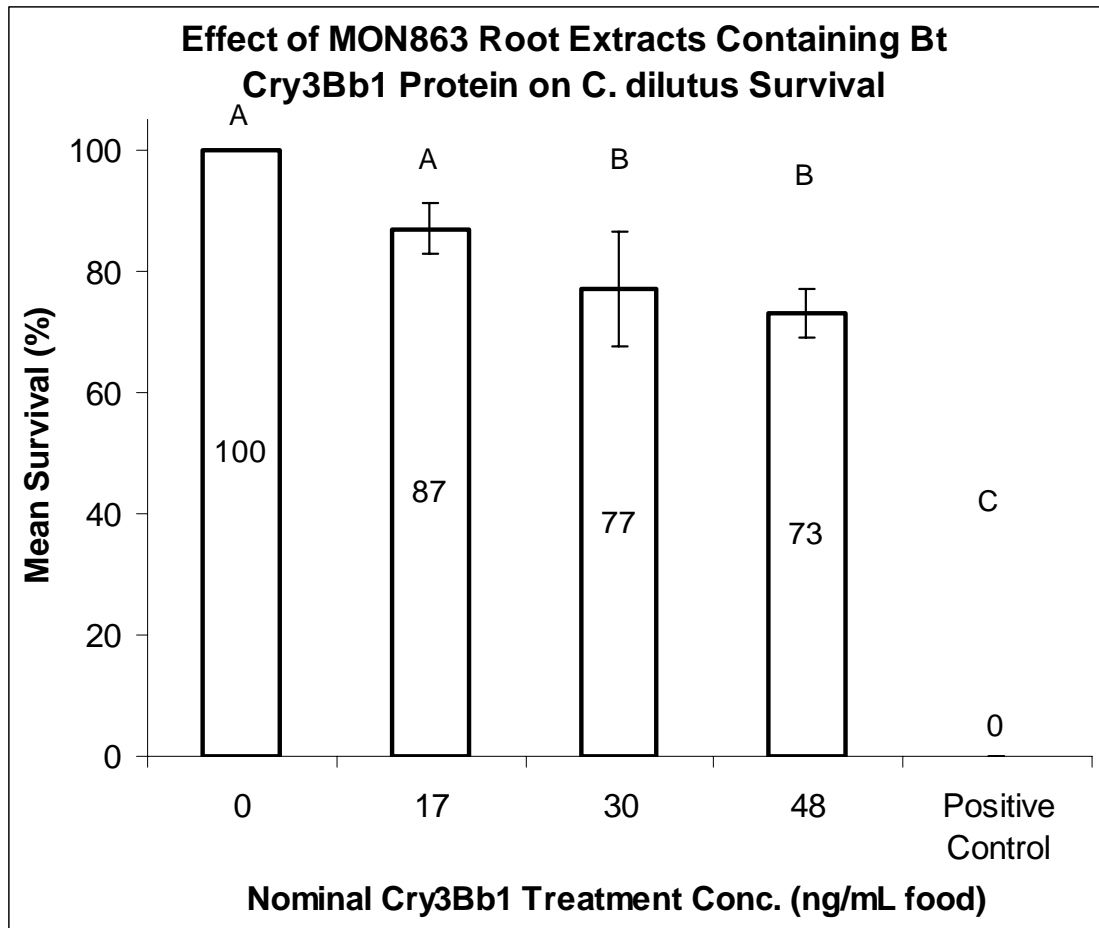


Figure 3. Average survival (\pm SE) of *C. dilutus* larvae exposed to Bt Cry3Bb1 protein present in MON 863 root extracts. Treatments include 0 (negative control), 17 (low), 30 (medium), and 48 ng/mL (high) Bt Cry3Bb1 added to TetraFin Slurry. The positive control was the orally active insecticide diflubenzuron at 20 ng/mL added to TetraFin Slurry. N = 6 replicates per treatment. Means with no common letter differ significantly ($p < 0.05$).

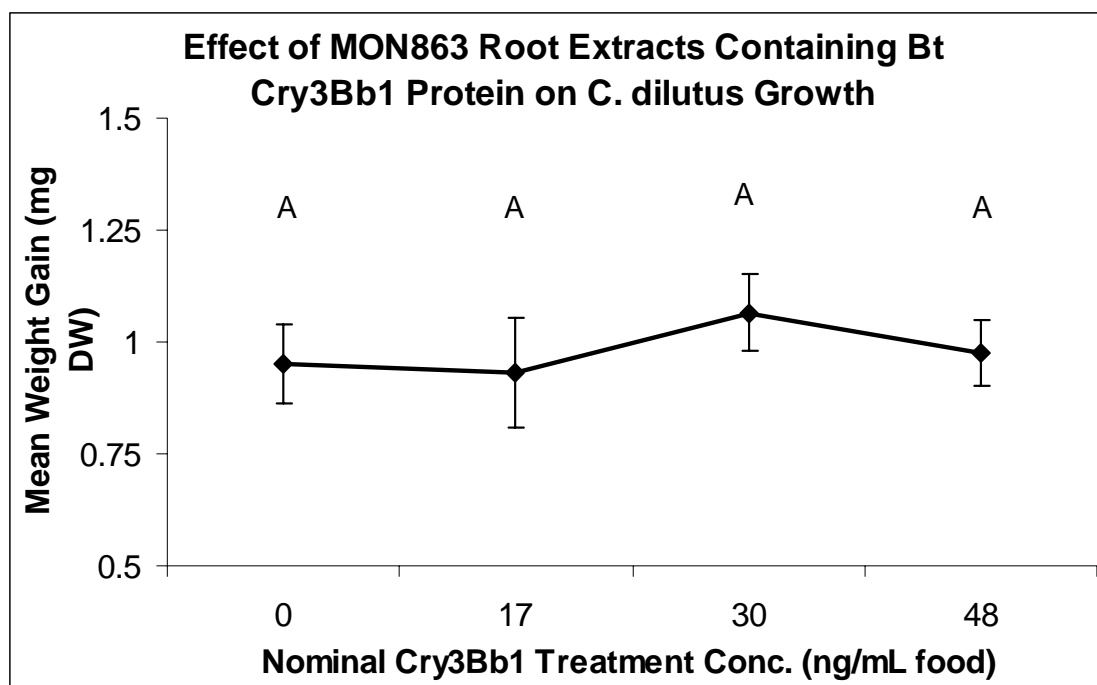


Figure 4. Average growth (\pm SE) of *C. dilutus* larvae exposed to Bt Cry3Bb1 protein present in MON 863 corn root extracts. Treatments include 0 (negative control), 17 (low), 30 (medium), and 48 ng/mL (high) Bt Cry3Bb1 added to TetraFin Slurry. N = 5 replicates per treatment. Means with no common letter differ significantly ($p < 0.05$).

Aerobic Aquatic Dissipation of Bt Cry3Bb1 in MON 863 Corn Leaf

$$f = a \cdot \exp(-b \cdot x)$$

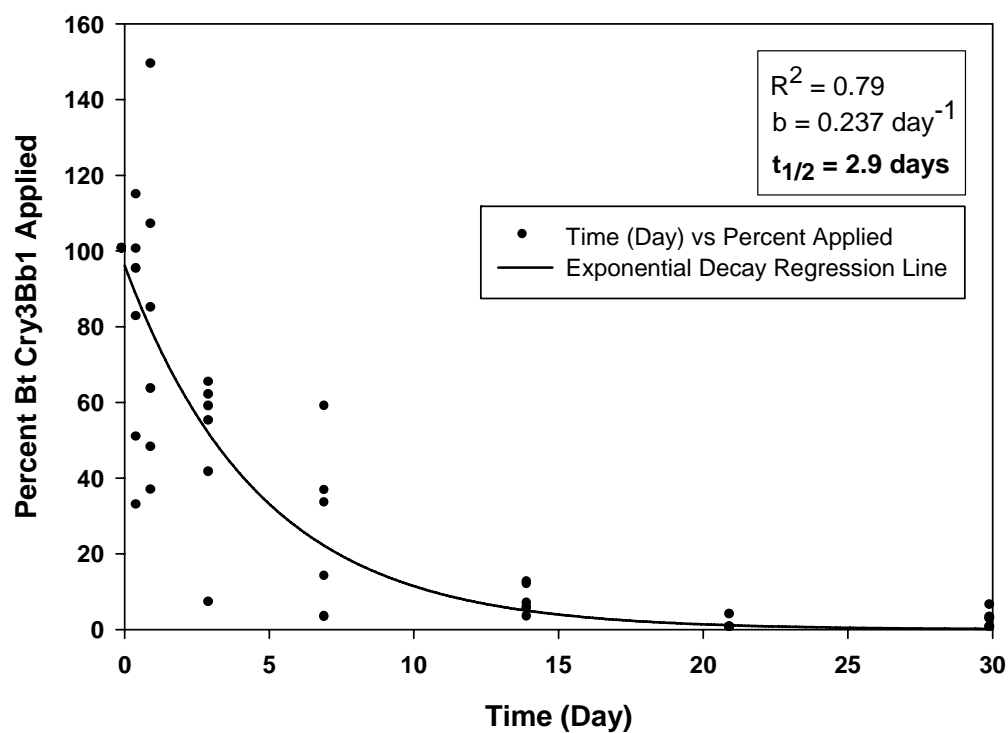


Figure 5. Nonlinear fit ($R^2=0.79$) of the aerobic aquatic dissipation of Bt Cry3Bb1 protein in MON 863 corn leaf. The parameter b ($0.237/\text{day}$) represents the first order rate constant. The aerobic aquatic half-life of Bt Cry3Bb1 in MON 863 corn leaf was 2.9 days.

Aerobic Aquatic Dissipation of Bt Cry3Bb1 in MON 863 Corn Stalk

$$f = a \cdot \exp(-b \cdot x)$$

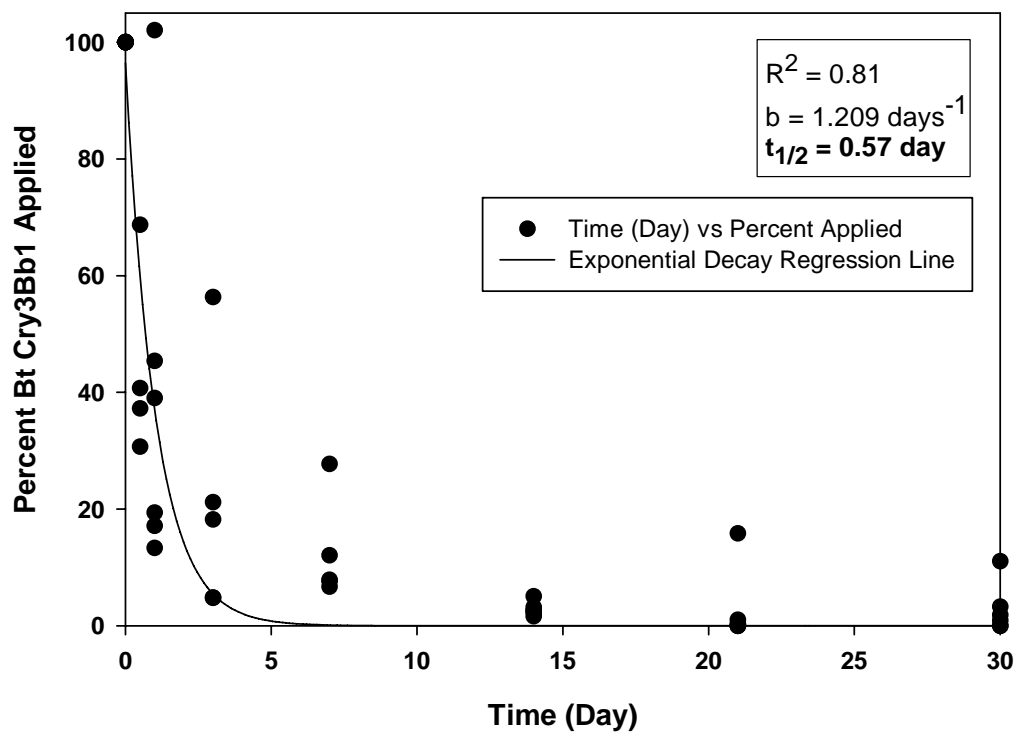


Figure 6. Nonlinear fit ($R^2 = 0.81$) of the aerobic aquatic dissipation of Bt Cry3Bb1 protein in MON 863 corn stalk. The parameter b (1.209/day) is the first order rate constant. The aerobic aquatic half-life of Bt Cry3Bb1 protein in MON 863 stalk was 0.57 day.

Aerobic Aquatic Dissipation of Bt Cry3Bb1 in MON 863 Corn Root

$$f = a \cdot \exp(-b \cdot x)$$

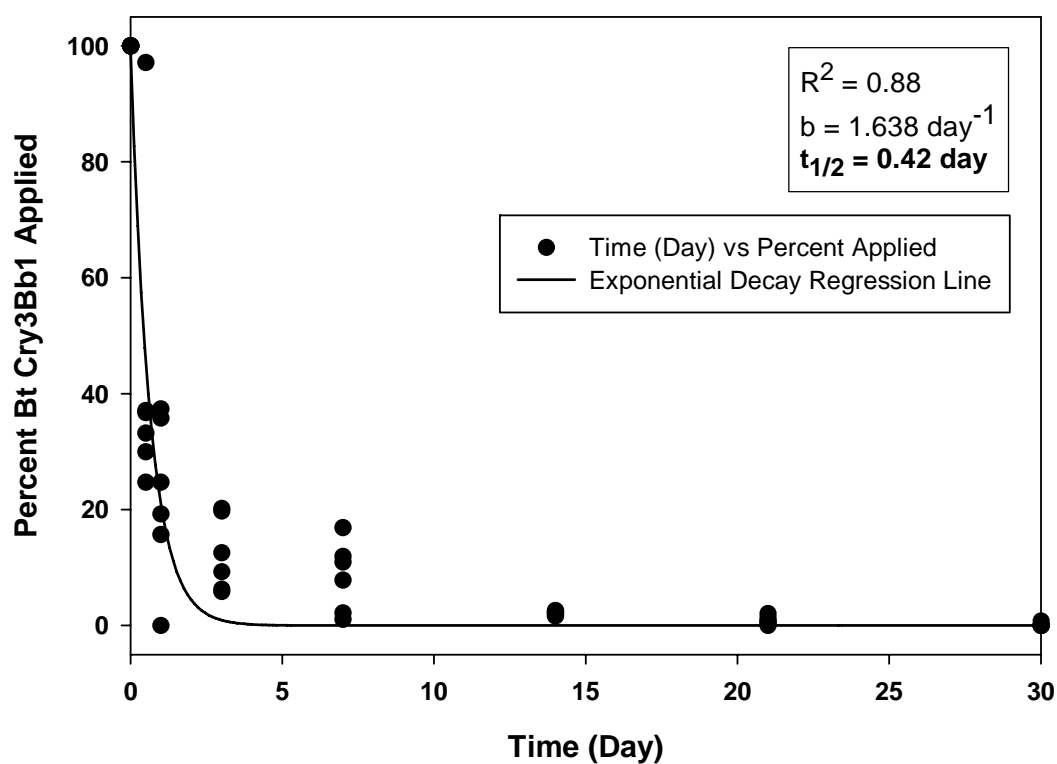


Figure 7. Nonlinear fit ($R^2 = 0.88$) of aerobic aquatic dissipation of Bt Cry3Bb1 in MON 863 corn root. The parameter b (1.638/day) is the first order rate constant. The aerobic aquatic half-life of Bt Cry3Bb1 in MON 863 corn root was 0.42 day.

CHAPTER 3. EXAMINATION OF THE FATE OF *BACILLUS THURINGIENSIS* (Bt) Cry3Bb1 PROTEIN IN A SOIL MICROCOSM

Abstract

Transgenic crops expressing insecticidal *Bacillus thuringiensis* (Bt) proteins have in-plant protection against a variety of agricultural pests. Understanding the environmental fate of transgenic Bt proteins is important because fate will dictate frequency, duration, and concentration of exposure to non-target organisms. There is little information in the peer-reviewed literature on the environmental fate of the coleopteran-active Bt Cry3Bb1 protein. In addition, the significance of macrodecomposing organisms, such as earthworms, isopods, and springtails, to the dissipation of Bt proteins from biotech corn has not been assessed. This laboratory microcosm study was conducted to determine the fate of Bt Cry3Bb1 in decomposing MON 863 corn residue and in soil. In addition, the importance of macrodecomposing organisms to the degradation of Bt proteins in corn residue was assessed. Laboratory microcosms containing MON 863 corn leaf, root, and stalk with and without macrodecomposers were used to examine the fate of Bt Cry3Bb1 in soil. A half-life of less than five days was found for Bt Cry3Bb1 protein in decomposing MON 863 corn residue. There was a trend of increasing half-life of Cry3Bb1 in microcosms containing macrodecomposers, however, this trend was only significant ($p < 0.05$) for Bt Cry3Bb1 in MON 863 leaf tissue and this increase is not likely relevant to non-target toxicity. There was only qualitative recovery of Bt Cry3Bb1 protein from soil extracts, which resulted in an approximate maximum concentration of 9 ng/g, translating to approximately 21 mg/ha Bt Cry3Bb1 from decomposing MON 863 post-harvest corn residue in the soil of an agricultural field. A low extraction efficiency of Bt Cry3Bb1 in soil contributed to the uncertainty in quantifying the input of the protein to soil in this study.

Introduction

The rod-shaped, aerobic, gram-positive bacterium *Bacillus thuringiensis* (Bt) is ubiquitous in the environment. This bacterium is characterized by its unique ability to produce insecticidal, crystalline (Cry) proteins during sporulation. These Cry proteins contain δ -endotoxins, which are active against a variety of insect orders, depending upon the Bt subspecies/strain that produces them. The insecticidal properties of Bt have been known

for almost a century, however, it wasn't until 1961 that the United States began to register pesticides containing Bt spores for the control of black fly, mosquito, and other aquatic larvae of biting pests [1]. In 1996, the first Bt crops became commercially available. These crops were engineered with the gene (*cry* gene) that is responsible for the production of insecticidal Cry proteins, thereby providing the crop with in-plant protection against a number of specific pests within a certain insect order. Since 1996, worldwide usage of Bt crops, especially Bt corn, has increased dramatically from 1.1 million hectare (ha) planted in 1996 to 19 million ha planted in 2006 [2].

The Bt protein used in the present studies was Bt Cry3Bb1, which is produced in YieldGard® Rootworm corn, and registered by the Monsanto Company (St. Louis, MO). This protein is coleopteran-active, and is specifically targeted for protection against several species of corn rootworm (*Diabrotica* spp.). YieldGard® Rootworm corn (transformation event MON 863, vector insert ZMIR13L) was registered in 2003 (http://www.epa.gov/pesticides/biopesticides/ingredients/tech_docs/cry3bb1/1_%20cry3bb1_exec_summ.pdf), and was chosen for this study because it is relatively new to the agricultural market, and because corn rootworm is an economically important pest. In addition, the U.S. Environmental Protection Agency registered YieldGard® Plus corn in 2005, which expresses both the Bt Cry3Bb1 protein and the lepidopteran-active Bt Cry1Ab protein (http://www.epa.gov/pesticides/biopesticides/ingredients/factsheets/factsheet_006430-006484.htm). This stacked-trait corn event, along with the economic importance of MON 863 corn, ensures the continual increased usage of the Bt Cry3Bb1 protein.

Understanding the terrestrial fate of Bt proteins is important because fate dictates the frequency, duration, and concentration of Bt proteins in the environment that may be available for exposure of non-target organisms. There are several routes by which Bt proteins can enter the soil, including the sloughing of root cells, the possible release of exudates from roots, and post-harvest plant residues. There is little information in the peer-reviewed literature regarding the environmental fate of Bt Cry3Bb1 protein. However, there have been several laboratory and field studies conducted using other Bt proteins (from genetically modified *E. coli* and field crops), from which conclusions regarding Bt Cry3Bb1 can be inferred.

Previous studies have shown that the dissipation of Bt Cry proteins in soil is biphasic. Palm et al. [3] showed a biphasic pattern of degradation for Cry1Ab protein added as crystals or in transgenic cotton tissue. Cry1Ab concentrations dropped from 225 ng/g initially to 30 ng/g after seven days in soil. By 30 days, the protein concentrations were still about 10 ng/g in soil. Donegan et al. [4] demonstrated Cry1Ab protein persistence up to 56 days in soil.

Later, Sims and Ream [5] evaluated the persistence of Cry2A protein, introduced to soil in transgenic cotton tissue, by an insect bioassay. They found the time required for 50% dissipation (DT_{50}) to be 15.5 and 31.7 days under laboratory and field conditions, respectively. However, dissipation slowed after the initial decrease in bioactivity, and approximately 20% of the bioactivity remained at 120 days. Sims and Ream [5] projected that approximately 486 g/acre (4 μ g/g soil) of Bt protein would be added to soil from a mature transgenic cotton crop, but this remains one of the few quantitative estimates of Bt protein loads to soil, and it was based solely on inputs from above-ground plant biomass.

Stotzky and coworkers conducted several studies on the soil fate of Bt proteins, particularly Bt Cry1Ab protein produced by *Bacillus thuringiensis kurstaki* (Btk). They found that Bt proteins bind readily to surface-active particles in soil (e.g. clay minerals and humic acids), do not easily desorb from these particles, and retain some of their biological activity while adsorbed. This rapid adsorption to surface-active particles reduces the availability of Bt proteins to microbial degradation, thereby increasing their persistence. In one study, purified Cry1Ab protein that was added to nonsterile soil was detected by insect bioassay 234 days after addition, which was the longest time point studied [6]. It was observed that Bt corn residue broke down at a slower rate than conventional corn biomass [7]. The higher lignin content in this Bt corn transformation event may have caused slower microbial decomposition, therefore, increasing the persistence of the Bt crop residue itself [8]. In addition, Cry1Ab was found to be released from root exudates of corn grown in hydroponic culture, sterile soil, and nonsterile soil [9]. Bt Cry1Ab protein was found in rhizosphere soil of 13 Bt corn hybrids, which represented three transformation events [10].

Two recent field studies have examined the soil fate of Cry1Ab protein present in transgenic corn. Zwahlen et al. [11] examined the degradation of Cry1Ab protein from Bt11 corn in representative tillage and no-tillage systems. In an eight-month study, Cry1Ab protein present in corn residue buried in litter bags was detected by enzyme-linked immunosorbent assay (ELISA) at 1.5% of the original protein concentration after seven months. In addition, Cry1Ab protein present in Bt11 corn residue on the soil surface was detected by ELISA at 0.3% of the original protein concentration after 200 days. In a study by Baumgarte and Tebbe [12], the fate of Cry1Ab protein present in Mon810 corn grown at two field sites was studied for two growing seasons. In both bulk and rhizosphere soil there were three to seven times greater concentrations of Cry1Ab protein detected by ELISA in the second growing season when compared to the first. In addition, Cry1Ab protein was detected in both the root and leaf residue present on the soil surface prior to second season planting. This study suggests an accumulation of Bt proteins from one growing season to the

next. Conflicting results were found in another study by Dubelman et al. [13]; the accumulation and persistence of Bt Cry1Ab protein from MON810 and Bt11 corn was examined in plots in five corn-producing states after three consecutive Bt corn growing seasons. Both post-anthesis and post-harvest soil samples (15 per plot) collected from all five Bt field plots had no effect on growth of larvae of the European corn borer. The results from this study, contrary to the study done by Baumgarte and Tebbe, indicate no accumulation or persistence of toxic levels of Bt Cry1Ab after three consecutive growing seasons of Bt corn.

In the only published study on the environmental fate of Bt Cry3Bb1, two out of three fields had no measurable Bt Cry3Bb1 protein in soil samples that were taken near-plant and between-row for three consecutive years during the growing season of MON 863 corn. The third field had lower clay content (5% compared to 36% clay), therefore, a higher extraction efficiency of Bt Cry3Bb1 was found for this soil in a spike-and-recovery study. The near-plant soil samples taken from the third field had a measured concentration of Bt Cry3Bb1 from 3-7 ng/g on all sampling occasions [14]. The lack of Bt Cry3Bb1 found in the soil samples collected from two of the three fields sampled may be due to a rapid degradation of Bt proteins in soil, or it may be due to an inability to extract Bt proteins that are adsorbed to surface-active particles in soil, such as clay.

One potentially important route of dissipation of Bt proteins within post-harvest crop residue is degradation via macrodecomposers. Unlike traditional insecticides, which are often degraded biologically through microbial action, plant-incorporated insecticides may be degraded biologically by both microbes and macrodecomposers, such as earthworms, terrestrial isopods, and springtails. In this study, the earthworm *Eisenia fetida*; the isopods *Trachelipus rathkii*, *Porcellio scaber*, and *Armadillidium* spp.; and the springtail *Folsomia candida* were used to evaluate the significance of macrodecomposing organisms on the degradation of Bt proteins present in post-harvest crop residue. Earthworms ingest soil, and derive nutrients from the microorganisms and organic matter contained in the ingested soil [15]. While the species that is typically used in toxicity testing and was used in the present study, *Eisenia fetida*, does not inhabit agronomic soils, its ecological role is similar to the earthworms that do inhabit these areas. Isopods are one of the most ecologically important decomposing organisms in the terrestrial ecosystem. They are saprophagous animals that feed on dead plant material, and break it down to a size that would be available to smaller decomposers, such as springtails and mites. In addition, they are found in a variety of habitats worldwide, including agricultural fields [16]. Springtails are the most numerous

insect in terrestrial ecosystems, and contribute to the decomposition process by feeding on the microfaunal growth present on decaying organic matter [17].

The terrestrial fate of Bt proteins is a key parameter governing exposure of non-target organisms in the environment, because the protein will be incorporated into soil with sloughing of root cells, possibly through the release of exudates from roots, and with plant tissue post-harvest. Therefore, it is important to establish laboratory methods that result in an accurate estimation of soil half-life for Bt proteins within biotech crops. The significance of macrodecomposers to the biological degradation of Bt proteins has not been established, despite the importance these organisms have in the degradation of post-harvest crop residue on agricultural fields. In addition, the input of Bt proteins to soil from the decomposition of plant residue has not been assessed. This may be an important route of entry for Bt proteins into terrestrial environments, and may dictate exposure to non-target organisms in terrestrial and aquatic environments. Therefore, the objectives of this study were to (1) obtain a half-life for Bt Cry3Bb1 protein present in MON 863 corn leaf, stalk, and root; (2) obtain a soil half-life for Bt Cry3Bb1; (3) determine the significance of macrodecomposing organisms to the degradation of Bt proteins in corn residue present in laboratory microcosms; (4) determine the input of Bt proteins to soil from decomposing corn residue.

Materials and Methods

Corn Material

Seeds of event MON 863 corn, transgenic line TP5504-TD, and near-isoline (nearest genetic equivalent) conventional corn line EXP258B were obtained from the Monsanto Company (St. Louis, MO). Seeds were planted in 19-L plastic buckets (one seed per bucket) containing Sun Gro® Sunshine LC1 Professional Mix potting soil (Sun Gro Horticulture Distribution, Bellevue, WA, USA). Plants were grown in a greenhouse on a 16 hour light:8 hour dark photoperiod at 27°C (light) and 20°C (dark), and were watered two to three times weekly. Plants were fertilized once, two months after germination, with Miracle-Gro® (The Scotts Company, Marysville, OH, USA) at the label-directed rate. The plants were harvested at tasseling (VT stage), which is the stage during which the plant tissue expresses the greatest concentration of Bt protein. Although corn plants are typically harvested after maturity is reached (R6 stage) and senescence has begun, a worst-case estimation of Bt Cry3Bb1 half-life may be useful for environmental risk assessment purposes, thus the plants were harvested during the life stage with the highest tissue concentration of Bt Cry3Bb1 and at the time when the tissue is most resistant to decomposition (i.e., anthesis). Prior to use in the study,

the isoline and MON 863 corn leaf, stalk, and root were cut into $\sim 3 \text{ cm}^2$ pieces that were representative of post-harvest crop residue.

Soil Description

Soil was collected from a reference field at the Iowa State University Agricultural Engineering/Agronomy Farm, located approximately 4 miles west of Ames, IA. The reference field was chosen because it is known to have not had pesticide application for greater than 30 years. Soil properties were analyzed by Midwest Laboratories, Inc. (Omaha, NE) using standard protocols. The soil was classified as a composite of Nicollet and Webster (mesic Endoaquolls), and as having a sandy loam texture. The organic matter content was 2.1%, pH was 6.7, and field moisture capacity was 16.7% (w/w). Following collection, the soil was sieved through a 2.83-mm mesh sieve to remove rocks and large detritus, and the soil was stored moist at 25°C in the dark to preserve the existing microbial community.

Bt Cry3Bb1 Quantification

Prior to the start of the study, the soil was extracted to determine whether background concentrations of Bt Cry3Bb1 were present. The soil was extracted three times with 10-mL (30-mL total) of a pH 10.5, high-salt buffer [3] with shaking at 300 rpm for one hour on an orbital shaker. The extracts were centrifuged at 3000 x g for 10 min. and the supernatant from each of the three extractions was pooled for analysis. The pH 10.5, high-salt buffer is the only buffer reported in the literature to be effective for extraction of Bt Cry3Bb1 protein from environmental matrices [14]. Bt Cry3Bb1 was measured in MON 863 and near-isoline corn leaf, root, and stalk prior to the start of the study. This was done by homogenizing three 20-mg samples of each corn tissue type from each corn line for one minute in 1-mL phosphate-buffered saline with Tween-20® (PBST) with a Delta® 9" bench-top drill press (Delta Machinery, Jackson, TN, USA). Extracts were allowed to settle for ~ 15 min., and the supernatant was removed for analysis. In addition, Bt Cry3Bb1 in corn tissue samples throughout the duration of the study were homogenized in this manner. Bt Cry3Bb1 was extracted from the whole-bodies of earthworms and isopods at the end of the study using the previously described method of corn tissue homogenization. Bt Cry3Bb1 concentration in extracts of MON 863 and isoline corn leaf, stalk, and root, as well as soil, earthworms, and isopods were analyzed using a Cry3Bb1 enzyme-linked immunosorbent assay (ELISA) kit (EnviroLogix; Portland, ME), and closely following the manufacturer's instructions. The qualitative kit was made quantitative with the use of a standard curve. Purified (83% pure as determined by SDS-PAGE) *E. coli*-produced Bt Cry3Bb1.11098 (Q349R) protein, provided

by Monsanto Company (St. Louis, MO) was used to generate a standard curve. The standard curve was as follows: 20 ng/mL, 10 ng/mL, 5 ng/mL, 3 ng/mL and 1.5 ng/mL. In addition, a buffer blank, negative control (isoline corn extract), and positive control (MON 863 corn extract) was run for each plate analyzed. Absorbance (optical density) of samples analyzed with each kit was read against the standard curve at 450 nm (650 nm reference wavelength) on a THERMOmax microplate reader and quantified with SOFTmax software (Molecular Devices; Sunnyvale, CA). Plates having a standard curve R^2 of less than 0.90, or a variability of greater than a 10% CV value were re-analyzed.

Significance of Decomposers to Soil Dissipation of Bt Proteins

This study was conducted in laboratory microcosms consisting of 1-L mason jars with wire mesh covers. Each microcosm contained 500 g of sieved soil and 5-g total (1% w/w) of processed near-isoline or MON 863 corn leaf, stalk, and root (i.e., ~1.6 g of each tissue type from each corn line). The corn residue was incorporated into the top 2 cm of the microcosm. The soil was brought to field moisture capacity (16.7%, w/w) for optimal microbial growth using distilled water, and this moisture level was maintained throughout the duration of the study using a Rapitest® Light and Moisture Meter (Luster Leaf Products, Inc.; Woodstock, IL). Treatments consisted of (1) near-isoline corn residue (conventional corn line control) without macrodecomposers, (2) MON 863 corn residue without macrodecomposers, and (3) MON 863 corn residue with the addition of macrodecomposers. The third treatment contained one earthworm (*Eisenia fetida*, 0.3-0.8 g each), five isopods (*Armadillidium* spp., *Trachelipus rathkii*, and *Porcellio scaber*; 47-197 mg each), and ten springtails (*Folsomia candida*, no weight obtained). These organisms were cultured for 1 to 4 years prior to the start of the study, using standard methods [15,16,17]. There were six time points used in this study (0, 0.5, 5, 10, 26, and 54 days), with three replicates of each treatment at each time point. The microcosms were maintained at 25°C with a 16 hour light:8 hour dark photoperiod. At each time point, three replicates of each of the three treatments were completely removed from the study. The corn leaf, stalk, and root were removed individually from each microcosm and washed with distilled water. There was no stalk material remaining in any of the treatments at Day 54. While the root material remained relatively whole throughout the duration of the study, the leaf material did not remain intact. One 20-mg sample (fresh weight) of each tissue type from each replicate at each time point was weighed, and frozen at -80°C until homogenization and analysis. On Day 54 of the study, the earthworm (N = 1 for all replicates) and isopods (N = 3-7) were removed from

each of the Day 54 microcosms in the third treatment and frozen at -80°C until homogenization and analysis.

Input of Bt Cry3Bb1 Protein to Soil from Decomposing Post-Harvest Corn Residue

Laboratory microcosms were used to investigate the input of Bt Cry3Bb1 protein to soil from decomposing corn residue post-harvest. Microcosms consisted of 250-mL French squares, containing 200 g sieved soil and 2 g (1% w/w) processed isoline or MON 863 corn leaf, stalk, and root incorporated into the top 2 cm of the microcosm. The soil was brought to optimal field moisture capacity (16.7%, w/w) using distilled water, and this moisture level was maintained throughout the duration of the study using a Rapitest® Light and Moisture Meter. There were two treatments utilized (1) microcosms containing near-isoline corn residue (conventional corn line control) and (2) microcosms containing MON 863 corn residue. Macrodecomposers were not utilized in this study. There were six time points (0, 0.5, 1, 5, 10, and 25 days), with three replicates of each treatment at each time point. The Day 0 time point was sampled at 2 hours after the addition of corn material. The microcosms were maintained at $24 \pm 1^\circ\text{C}$, with a photoperiod of 16 light hours:8 dark hours. At each time point, three replicates from each treatment were completely removed from the study. The soil from the top and bottom half of the microcosm was removed, and sieved using a 2-mm mesh sieve. The corn leaf, stalk, and root was removed, washed with distilled water, and air-dried. A 20-mg sample of leaf, stalk, and root from each replicate was weighed and frozen at -80°C until homogenization and analysis. A 5-g sample of the sieved soil from the top and bottom half of the microcosm was weighed and frozen at -80°C until extraction and analysis.

Statistical Analysis and Design

Each study was conducted as a completely randomized design, due to the uniform conditions present in the environmental chambers where the studies took place. Half-lives for Bt Cry3Bb1 protein in MON 863 corn leaf, stalk, and root from each treatment in the macrodecomposer study and the soil fate study were calculated based on mean percent Cry3Bb1 remaining at each time point according to first-order degradation kinetics using JMP software (v. 6.0). SigmaPlot (v. 10.0) was used to graph the data. The nonlinear model, $f = a \cdot \exp(-b \cdot x)$, was used to fit the data for each tissue type in each treatment. For this model, f is the response variable (percent Cry3Bb1 applied), the parameter a determines the asymptotes of the curve, b is the first order rate constant, and x is the time in days. The

dissipation curve for each MON 863 tissue type was compared for the no-decomposer and the decomposer-added treatments using JMP. Differences between treatments for the dissipation curve of each tissue type were determined by calculating F-values with statistical significance defined at $p < 0.05$ [18].

Results

Bt Cry3Bb1 Quantification

There was no Bt Cry3Bb1 protein measured in the near-isoline corn leaf, stalk, and root tissues prior to the start of the decomposer study or the soil fate study. Additionally, no Bt Cry3Bb1 was measured in the near-isoline corn at any time point during either study. There was no Cry3Bb1 measured in the soil prior to the start of the decomposer study or the soil fate study, which confirmed that there was no background level of Bt Cry3Bb1 in the soil. ELISA analysis of the MON 863 corn leaf, stalk, and root prior to the start of each study resulted in the average Bt Cry3Bb1 concentrations at anthesis shown in Table 1. The concentration of Bt Cry3Bb1 in MON 863 corn leaf ranged from 14-33 $\mu\text{g/g}$, MON 863 stalk ranged from 5-12 $\mu\text{g/g}$, and MON 863 root ranged from 89-104 $\mu\text{g/g}$.

Significance of Decomposers to Soil Dissipation of Bt Proteins

The dissipation curves for MON 863 corn leaf, stalk, and root in microcosms with and without macrodecomposers fit a first-order degradation kinetics model, with R^2 values for each curve greater than 0.99 [18]. The half-life of Cry3Bb1 protein in decomposing MON 863 corn residue was less than 5 days for all corn tissues and treatments analyzed. There was a trend of increasing half-life of Bt Cry3Bb1 protein in MON 863 corn residue in microcosms with macrodecomposers present as compared to the treatment containing MON 863 corn only. This trend was only significant for the Cry3Bb1 protein in MON 863 leaf tissue (Table 2). The first-order half-life for Bt Cry3Bb1 in MON 863 corn leaf in the treatment without decomposers was 1 day, while the half-life in the treatment with decomposers added was 4 days. The first-order half-life of Cry3Bb1 protein in MON 863 corn stalk was 1 day in the treatment without macrodecomposers, and 2 days for the treatment with decomposers. The first-order half-life of Cry3Bb1 in MON 863 corn root in the treatment without macrodecomposers was 0.23 day, while the half-life in the treatment with decomposers was 0.24 day. There was no treatment effect on the dissipation of Cry3Bb1 protein in MON 863 stalk or root residue, therefore, the data from these two

treatments were pooled and the resulting half-life was estimated for Bt Cry3Bb1 protein from the first-order rate constant of the nonlinear regression line (Figures 1 and 2).

Although it was not the goal of this study to determine the effects of Bt Cry3Bb1 protein on the macrodecomposers used, it was noted that all macrodecomposers added to the treatment survived and reproduction was observed for *F. candida* and the isopods (spp.). No Bt Cry3Bb1 protein was recovered from the whole-body extracts of the earthworms or isopods present in microcosms containing macrodecomposers from Day 54 of the study.

Input of Bt Cry3Bb1 Protein to Soil from Decomposing Post-Harvest Corn Residue

This study resulted in Bt Cry3Bb1 half-lives that were similar to those seen in the macrodecomposer study, and were estimated using a first-order degradation kinetics model. The overall half-life of Bt Cry3Bb1 was less than 6 days for all MON 863 corn leaf, stalk, and root residue.

There was only qualitative recovery of Bt Cry3Bb1 protein from soil extracts taken from the top half of microcosms containing MON 863 corn residue on Days 0 and 0.5. That is, while color was clearly generated from these extracts the concentration of Bt Cry3Bb1 could not be quantified because it fell below the limit of detection of the ELISA (below 1.5 ng/mL). Therefore, it can only be concluded that the concentration of Bt Cry3Bb1 in these extracts was above 0 ng/mL and below 1.5 ng/mL, or above 0 ng/g and below 9 ng/g soil. No Bt Cry3Bb1 was measured in the top half of microcosms containing MON 863 corn residue at any time point after Day 0.5. In addition, no Bt Cry3Bb1 was measured in any of the soil extracts from the top or bottom half of microcosms containing isoline corn residue, or from any of the soil extracts from the bottom half of microcosm containing MON 863 corn residue at any time point.

Discussion

In this study, Bt Cry3Bb1 protein in decomposing MON 863 corn leaf, stalk, and root tissue was found to decay at an exponential rate and fit a first-order degradation kinetics model. A short half-life for Bt Cry3Bb1 protein in MON 863 corn tissue was found in the present studies, with a half-life of less than 6 days for leaf, root, and stalk residue in the macrodecomposer study and the soil fate study. At the end of 25 days, less than 1% of the Bt Cry3Bb1 protein remained in MON 863 corn leaf, stalk, and root in both studies. Because of the low recovery of Bt Cry3Bb1 from soil in the second study, a half life could not be

estimated and it is not known whether the dissipation would have been exponential or biphasic in this matrix.

In the first study, which examined the significance of macrodecomposing organisms to the dissipation of Bt Cry3Bb1 in MON 863 corn tissues, a trend of increasing Cry3Bb1 half-life in those treatments containing macrodecomposers was found. Although this trend was only statistically significant for MON 863 leaf tissue, these results are consistent with the results of a study done by Sims and Ream [5]. This study found that the 50% dissipation time (DT_{50}) of Bt Cry2A protein in transgenic cotton in field studies was approximately two times greater than the half-life found in the laboratory, where no macrodecomposers were present. The reasons for this trend of increasing half-life in the presence of macrodecomposers is not yet known, but may be due to a decrease in the microbial and fungal activity in the system due to microbial and microfaunal grazing by earthworms, isopods, springtails, and other decomposing organisms. While there was a significantly increased half-life of Bt Cry3Bb1 protein in MON 863 corn leaf in the treatment containing macrodecomposers, this increase is likely not ecologically/toxicologically relevant, as a half-life of 1 day versus 4 days still constitutes acute exposure. Therefore, it may not be necessary to include macrodecomposing organisms in laboratory investigations on the terrestrial fate of Bt proteins, however, the significance of macrodecomposers to Bt protein degradation in the field may be much more pronounced than in the lab. There was no Bt Cry3Bb1 measured in the whole-body extracts of earthworms and isopods on the final day of the first study. More success may have been achieved by measuring the protein in a more concentrated state; hence by dissecting out the guts of these organisms and extracting the protein out of these tissues the protein may have been within the ELISA's limit of detection.

In the second study, which examined the input of Bt Cry3Bb1 to soil from decomposing MON 863 corn leachates, similar half-lives were seen for Cry3Bb1 to the first study. Bt Cry3Bb1 protein was only qualitatively recovered from the microcosm soil on Days 0 (~ 2 hours after microcosm addition) and Day 0.5, resulting in an estimation of Bt Cry3Bb1 protein soil concentration from decomposing MON 863 corn of 0-9 ng/g or approximately 4.5 ng/g. Using the maximum, 9 ng/g, as an estimate of Bt Cry3Bb1 input to soil from decomposing MON 863 corn, as well as the estimate that following harvest there is approximately 2-2.5 metric tons (t)/ha corn crop residue left on or in the soil [11], there would be approximately 21 mg/ha Bt Cry3Bb1 from decomposing MON 863 post-harvest corn residue.

The low recovery of Bt Cry3Bb1 from the microcosm soil in the second study is likely due to the inadequacy of the current analytical method, specifically the extraction buffer. In

a soil spike-recovery study, a 41% recovery of Bt Cry3Bb1 protein from autoclaved soil was achieved after immediate extraction following pure protein spiking using a high-salt, high-pH buffer. Twenty-four hours after spiking autoclaved soil with pure Bt Cry3Bb1, only a 24% recovery resulted possibly due to the rapid adsorption of these proteins to surface-active particles in soil (unpublished data, not presented). In order to more accurately study the terrestrial and aquatic fate of Bt proteins from transgenic crops, it is necessary to develop extraction buffers that are able to effectively extract these proteins from environmental matrices after soil adsorption occurs. In addition, it was found that Bt proteins active against different insect orders (e.g., Cry1F vs. Cry3Bb1) may not produce similar recoveries from soil when using the same extraction buffer (unpublished data, not presented). This is a challenge facing all research on the environmental fate and effects of Bt proteins from transgenic crops, and it is important that more effort be put into improving the current analytical methods for these novel insecticides.

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Table 1. Average expression levels (\pm SE) of Bt Cry3Bb1 protein in MON 863 and EXP258B (near-isoline) corn leaf, stalk, and root at anthesis and senescence measured by enzyme-linked immunosorbent assay (ELISA).

Corn Type	Growth Stage	Tissue Type	Bt Cry3Bb1 Concentration ($\mu\text{g/g}$)
MON 863	Anthesis	Leaf	24.6 ± 5.6
		Stalk	7.4 ± 2.2
		Root	96.3 ± 7.6
Near Isoline	Anthesis	Leaf	0
		Stalk	0
		Root	0

Table 2. Statistical summary for nonlinear regression of Bt Cry3Bb1 protein in MON 863 corn leaf residue in microcosms with and without macrodecomposers. Parameters a and b determine the asymptotes of the regression curve, and c and c1 describe the first-order rate constants for each treatment. Treatments without a common letter differ significantly ($p > 0.05$; Fisher's F-test).

Effect	a	b	c	c1	SSE	DF	F	P-value
Time	-1.042	99.994	0.231		21332.61	33		
Treatment ^a	0.436	103.172	0.718	-0.556	17389.77	32	6.099	0.0189

Treatment ^a	k, day ⁻¹	t _{1/2} , days
Leaf	c = 0.718	1 ^A
Leaf + Decomposers	c + c1 = 0.162	4 ^B

Degradation of Bt Cry3Bb1 from MON863 Corn Stalk Residue

$f = a \cdot \exp(-b \cdot x)$

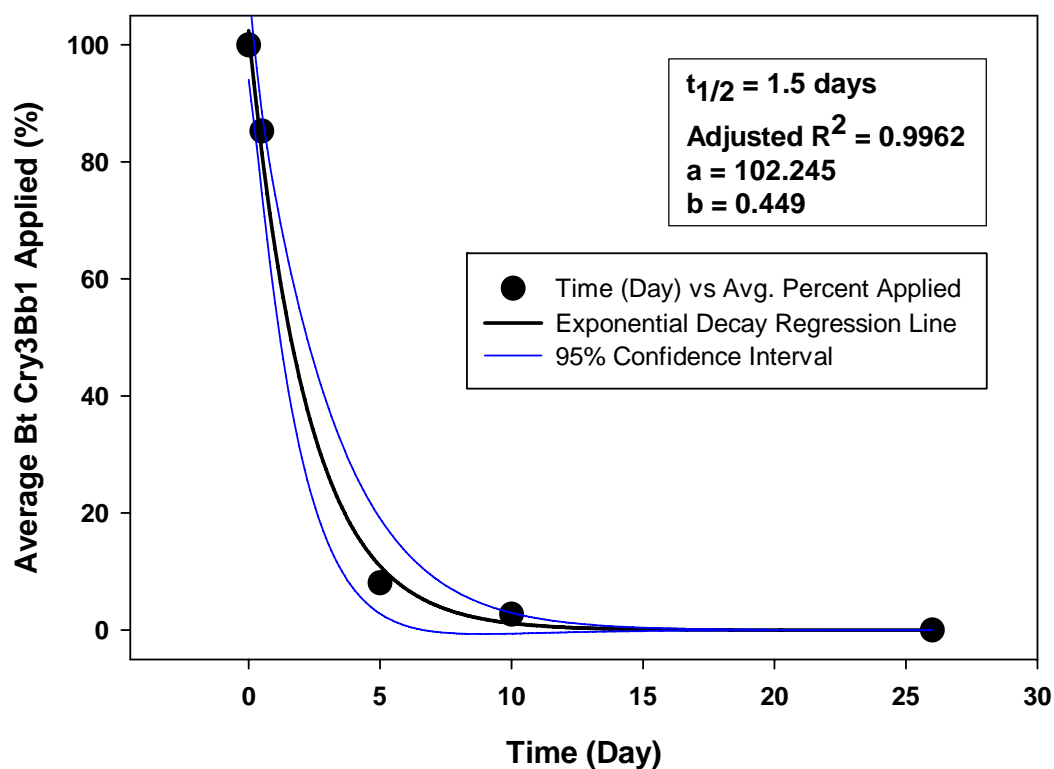


Figure 1. Dissipation of Bt Cry3Bb1 protein in MON 863 corn stalk residue in laboratory microcosms with and without macrodecomposers. No significant effect of macrodecomposers was found for these two treatments. The solid lines represent the 95% confidence interval for the pooled data from both treatments combined. Parameter a determines the asymptote of the curve, while parameter b is the first-order rate constant.

Degradation of Bt Cry3Bb1 from MON863 Corn Root Residue

$f = a \cdot \exp(-b \cdot x)$

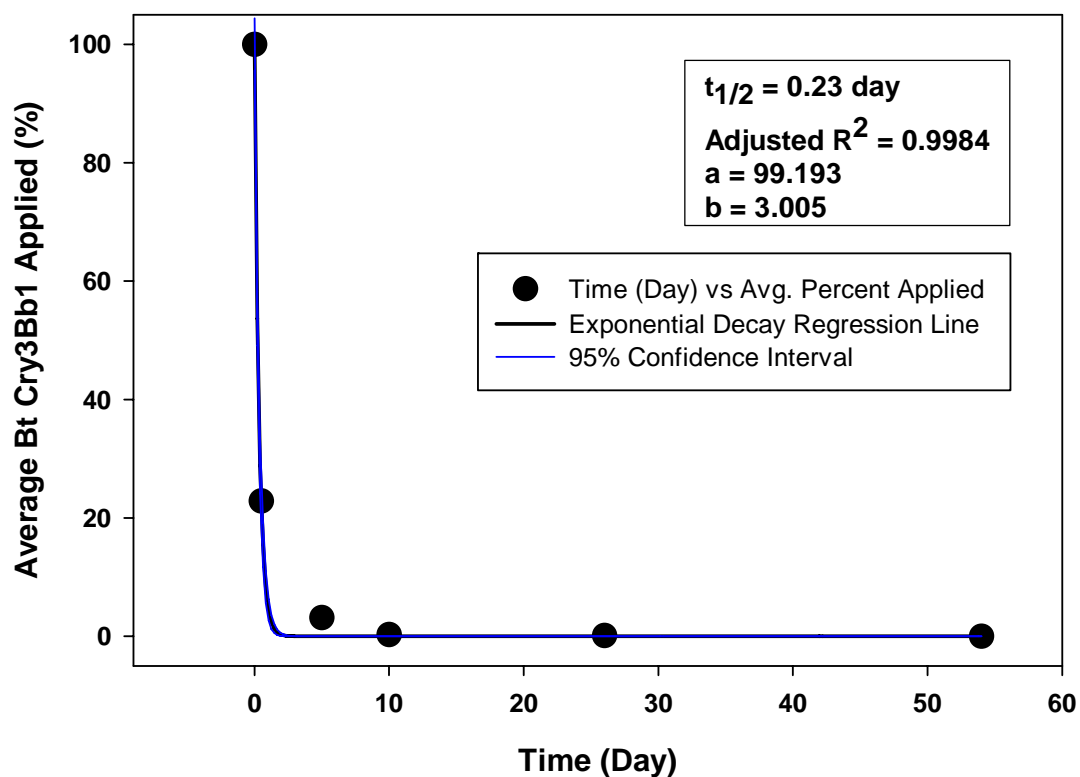


Figure 2. Dissipation of Bt Cry3Bb1 protein in MON 863 corn root residue in laboratory microcosms with and without macrodecomposers. No significant effect of macrodecomposers was found for these two treatments. The solid lines represent the 95% confidence interval for the pooled data from both treatments. Parameter a determines the asymptote of the curve, while parameter b is the first-order rate constant.

CHAPTER 4. EXAMINATION OF METHODOLOGIES TO IMPROVE EXTRACTION OF Bt PROTEINS FROM ENVIRONMENTAL MATRICES

Abstract

In 1996, crops expressing *Bacillus thuringiensis* (Bt) insecticidal crystalline (Cry) proteins were introduced to the agricultural market. The acreage on which these crops are grown has increased by 18 million ha in the last 10 years. The terrestrial and aquatic fate of transgenic Bt proteins are key parameters governing exposure of non-target organisms in the environment. Because of potential non-target effects on terrestrial and aquatic organisms, it is important to accurately quantify Bt protein exposure with environmental fate studies. However, conflicting results have been found for terrestrial fate studies, and there is almost no information on the aquatic fate of transgenic Bt proteins. The various results seen in the terrestrial fate studies are likely in part due to differences in the analytical methods used. Currently, there is no reliable and accurate analytical technique for quantifying transgenic Bt proteins from environmental matrices with high recovery efficiency. The goals of this study were to improve the extraction of Bt proteins from environmental matrices. A series of spike-and-recovery experiments using the lepidopteran-active Bt Cry1F and the coleopteran-active Bt Cry3Bb1 were conducted to determine the best extraction method for these two proteins. A differential extraction was found for these two classes of proteins, with the best-available buffer for Cry1F being a biomimetic buffer and the best-available buffer for Cry3Bb1 being a high-salt, high-pH buffer. Despite a good extraction efficiency of Cry1F from soil, the biomimetic buffer was not able to extract any Cry1F from the soil of field plots containing Herculex 1 corn. The results from this study indicate a need for extraction buffers that can overcome the adsorption of Bt proteins to surface-active particles in soil. Several solid-phase extraction (SPE) methods were attempted for cleanup and concentration of Bt proteins from environmental matrices. Although good retention to the SPE tubes was achieved using carbon-18 and strong-anion-exchange cartridges, an efficient method for eluting Bt proteins from the tubes was not achieved.

Introduction

Bacillus thuringiensis (Bt) is a rod-shaped, aerobic, gram-positive, spore-forming bacterium that is indigenous to many environments typically colonized by insects [1,2].

During sporulation, Bt produces crystalline (Cry) proteins that contain insecticidal δ -endotoxins. More than 3000 Bt strains have been isolated thus far, which are active against Lepidoptera, Diptera, Coleoptera, or both Lepidoptera and Diptera [3,4]. Since 1961, the U.S. has registered pesticides containing spores from a variety of *B. thuringiensis* strains for control of black fly, mosquito, and other aquatic larvae of biting pests [4]. In 1996, transgenic crops expressing Bt insecticidal proteins, thus containing in-plant protection against certain pest species, were introduced to the agricultural market. Since then, worldwide usage of Bt crops, especially Bt corn, has increased dramatically from 1.1 million hectare (ha) grown in 1996 to 19 million ha in 2006 [5]. The high worldwide adoption rates of Bt proteins has generated much attention from scientists researching the sustainability of these novel insecticides due to the decreased need for chemical insecticides that has resulted from the increased usage of Bt crops.

For this research the lepidopteran-active Bt Cry1F and the coleopteran-active Bt Cry3Bb1 proteins were used as models to examine methods to improve the analysis of transgenic Bt proteins from environmental matrices. *Bacillus thuringiensis* Cry1F protein (transformation event TC1507, plasmid insert PHI8999) expressed in transgenic corn is registered under the trade names Herculex® 1 Insect Protection and Pioneer Brand Seed Corn with Herculex® 1. It was registered in 2001 by both Mycogen Seeds (c/o Dow Agrosciences LLC; Indianapolis, IN) and Pioneer Hi-Bred International (Johnston, IA). Bt Cry1F is effective against lepidopteran pests of corn, such as European corn borer (ECB; *Ostrinia nubilalis*), southwestern corn borer (SWCB; *Diatraea grandiosella*), fall armyworm (FAW; *Spodoptera frugiperda*), and black cutworm (*Agrostis ipsilon*). The protein transformation event is registered until 2008 (http://www.epa.gov/pesticides/biopesticides/ingredients/tech_docs/brad_006481.pdf). Genetically modified corn expressing *Bacillus thuringiensis* Cry3Bb1 protein (transformation event MON863, vector insert ZMIR13L) was registered by Monsanto Company (St. Louis, MO) in 2003 under the trade name YieldGard® Rootworm Corn. Bt Cry3Bb1 is active against coleopteran pests of corn, particularly the corn rootworm (CRW), which includes the western corn rootworm (*Diabrotica virgifera virgifera*), northern corn rootworm (*D. barberi*) and Mexican corn rootworm (*D. virgifera zea*, http://www.epa.gov/pesticides/biopesticides/ingredients/tech_docs/cry3bb1/1_%20cry3bb1_exec_summ.pdf). Bt Cry1F and Cry3Bb1 proteins were chosen for these studies because they are relatively new to the agricultural market and provide protection against economically important pest species, which ensures their continual increased usage in the future.

The terrestrial and aquatic fate of Bt proteins is a key parameter governing exposure of non-target organisms in the environment. In terrestrial environments, Bt proteins are incorporated into soil with sloughing of root cells, possibly through the release of exudates from roots, and with plant tissue post-harvest. The binding, persistence, and movement of Bt proteins in soil will dictate the potential exposure of non-target species, including aquatic species. There are multiple conceivable routes of movement or transport by which Bt proteins could reach aquatic ecosystems. Bt proteins are thought to bind strongly to soil components, including clays. Because of this, they are likely to be transported while bound to the particulate matter (soil, decaying plant tissue) in runoff or eroded sediment. Additionally, Saxena et al. [6] reported that a portion of the Bt protein deposited in soil is downwardly mobile and detectable in the leachate from a soil column, and that the downward movement of Bt protein is highly dependent upon the soil texture. The vertical movement of Bt Cry1Ab protein decreased as the clay concentration of the soil increased, indicating that in high clay soils Bt proteins would be transported to aquatic systems via runoff and erosion. A potentially important aspect of movement of Bt proteins to aquatic systems is the movement of plant material such as leaf litter, pollen, and crop dust.

Because of potential non-target effects on terrestrial and aquatic organisms, it is important to accurately quantify Bt protein exposure with environmental fate studies. However, there is almost no information on the aquatic fate of transgenic Bt proteins, and studies conducted on their terrestrial fate show conflicting results. Some studies indicate a rapid soil dissipation, therefore, only acute non-target exposure. Other studies have shown an accumulation of Bt protein from one field season to the next. Bt Cry1F dissipation was found to fit a first-order, exponential decay model with an estimated half-life of less than one day [7]. Similarly, Sims and Holden [8] found a DT_{50} , or 50% dissipation time, of 1.6 days for Bt Cry1Ab protein from transgenic corn tissue. However, other studies have shown that the dissipation of Bt Cry proteins in soil is biphasic. Palm et al. [9] showed a biphasic pattern of degradation for Cry1Ab protein added as crystals or in transgenic cotton tissue. Cry1Ab concentration dropped from 225 ng/g initially to 30 ng/g after seven days in soil. After 30 days, the protein concentration was still about 10 ng/g. Donegan et al. [10] demonstrated Cry1Ab protein persistence up to 56 days in soil. Three recent field studies also show conflicting results for Bt protein dissipation in soil. Zwahlen et al. [11] examined the degradation of Cry1Ab protein from Bt11 corn in representative tillage and no-tillage systems. In an eight-month study, Cry1Ab protein present in corn residue buried in litter bags was measured at 1.5% of the original protein concentration after seven months. In addition, Cry1Ab protein present in Bt11 corn residue on the soil surface was found at 0.3%

of the original protein concentration after 200 days. In a study by Baumgarte and Tebbe [12], the fate of Cry1Ab protein present in Mon810 corn grown at two field sites was studied for two growing seasons. In both bulk and rhizosphere soil there were three to seven times greater concentrations of Cry1Ab protein in the second growing season when compared to the first. In addition, Cry1Ab protein was detected in both the root and leaf residue present on the soil surface prior to second season planting. This study suggests an accumulation of Bt proteins from one growing season to the next. Conflicting results were found in another study by Dubelman et al. [13]. The accumulation and persistence of Bt Cry1Ab protein from MON810 and Bt11 corn was examined in plots in five corn-producing states after three consecutive Bt corn growing seasons. Both post-anthesis and post-harvest soil samples (15 per plot) collected from all five Bt field plots had no effect on growth of larvae of the European corn borer. The results from this study, contrary to the study done by Baumgarte and Tebbe, indicate no accumulation or persistence of toxic levels of Bt Cry1Ab after three consecutive growing seasons of Bt corn.

The various results seen in terrestrial fate studies on transgenic Bt proteins may be due to soil type, environmental conditions, or the type of Cry protein used. However, each of these studies also differed in the analytical method used, which may also be an important source of variation. Currently, there is no reliable, accurate, and highly efficient analytical technique for quantifying Bt proteins from environmental matrices that can be used by many labs and for many matrices. The development of an analytical method that accurately quantifies biologically active (i.e., intact) Bt proteins from environmental matrices is currently an under-researched area. The two most widely used methods for quantification of Bt proteins from environmental matrices are bioassay and enzyme-linked immunosorbent assay (ELISA). Bioassay involves adding the matrix to be measured (e.g., soil containing Bt protein) to the food of a target insect pest. Survival or growth is compared to a known toxicity curve, generated with purified protein, to obtain a concentration of Bt protein in the matrix. Bioassays do not require the protein to be extracted from the matrix; however, this method does not provide a high degree of accuracy and may be adversely affected by the presence of other toxicants in the matrix. Bioassay is also an expensive and time-consuming method of Bt protein quantification. Unlike bioassay, ELISA is a very sensitive, efficient, and relatively inexpensive method of Bt protein quantification. However, ELISA does require that the Bt protein be extracted from the matrix, and extraction efficiency of Bt proteins is low because of the tendency of these proteins to adsorb to surface-active particles (e.g., clay and humic acids) in soil and sediment. The specificity of ELISA is a major

disadvantage, as ELISAs are able to quantify cleaved Bt protein fragments (i.e. not biologically active) present in environmental matrices.

The lack of an accurate analytical method is a challenge in determining Bt protein exposure to non-target terrestrial and aquatic organisms, which is an essential part of assessing the ecological risk of Bt proteins. The objectives of the present study were to (1) determine the best-available extraction method for quantification of Bt Cry1F and Bt Cry3Bb1 proteins from soil and sediment, (2) use the best-available extraction method to monitor Bt Cry1F in the soil of an agricultural field containing Herculex 1® corn, (3) determine whether Western blot or electron-spray ionization (ESI)-mass spectrometry (MS) can be used to confirm the presence of biologically active Bt proteins in soil and sediment extracts, and (4) to develop a solid-phase extraction (SPE) method for cleanup and concentration of Bt proteins from environmental matrices.

Materials and Methods

Spike-and-Recovery of Bt Cry1F and Bt Cry3Bb1 from Environmental Matrices

Soil Description

Soil for the spike-and-recovery studies was collected from a reference field at the Iowa State University Agricultural Engineering/Agronomy Farm, located approximately 4 miles west of Ames, IA. This reference field was chosen because it is known to have not had pesticide application for greater than 30 years. Sediment used in the spike-and-recovery studies was collected from a pond at the Iowa State University Horticulture Farm (Gilbert, IA). Soil and sediment properties were analyzed by Midwest Laboratories, Inc. (Omaha, NE) using standard protocols. The soil was classified as a Nicollet and Webster composite (mesic Endoaquolls), and as having sandy loam texture with 60% sand, 22% silt, and 18% clay. The organic matter content was 2.1%, pH was 6.7, and field moisture capacity was 16.7% (w/w). The sediment had 1.4% organic matter and a pH of 7.6, and was composed of 75.8% sand and 24.2% clay. Following collection, the soil was sieved through a 2.83-mm mesh sieve to remove rocks and large detritus. The soil and sediment were autoclaved at 121°C for 1 hour to reduce microbial degradation of Bt Cry1F and Cry3Bb1.

Bt Cry1F and Cry3Bb1 Quantification

Purified, *E. coli*-produced Bt Cry1F protein was provided by Dow AgroSciences (Indianapolis, IN). Purified (83% pure as determined by SDS-PAGE), *E. coli*-produced Bt Cry3Bb1.11098 (Q349R) protein was provided by Monsanto Company (St. Louis, MO). Bt Cry3Bb1 and Bt Cry1F concentration in soil extracts were analyzed using a Cry3Bb1 or

Cry1F ELISA kit (EnviroLogix; Portland, ME), and closely following the manufacturer's instructions. The qualitative kits were made quantitative with the use of a standard curve, as follows: 20 ng/mL, 10 ng/mL, 5 ng/mL, 3 ng/mL and 1.5 ng/mL. In addition, a buffer blank, negative control (isoline corn extract), and positive control (MON 863 or Herculex 1 corn extract) was run for each plate analyzed. Absorbance (optical density) of samples analyzed with each kit was read against the standard curve at 450 nm (650 nm reference wavelength) on a THERMOmax microplate reader and quantified with SOFTmax software (Molecular Devices; Sunnyvale, CA). Plates having a standard curve R^2 of less than 0.90, or a variability of greater than a 10% CV value were re-analyzed.

Bt Cry1F Spike-and-Recovery

Bt Cry1F recovery was measured from freshly-spiked and aged soil and sediment samples. A 0.2-g sample of soil or sediment was spiked with 100 μ L of a 375 ng/mL Cry1F solution, which resulted in a concentration of 187 ng/g Cry1F in both matrices. The control treatment was spiked with 100 μ L phosphate-buffered saline with Tween-20® (PBST), which was used to make the Cry1F-spiking solution. Samples were aged for 0.25, 24, 48, or 96 hours, and were maintained at 25° C at a 16 hr. light:8 hr. dark photoperiod. There were at least two replicates of each treatment at each time point, therefore, at the appropriate time point, corresponding samples were removed from the study and were extracted with 750 μ L biomimetic buffer [14], biomimetic buffer with 0.5 mg/mL bovine pancreatic trypsin, high-salt, pH 10.5 buffer [15], or high-salt, pH 10.5 buffer containing 0.5 mg/mL trypsin. A description of these buffers can be found in Table 1. Samples were placed on an orbital shaker for one hour, and were centrifuged at 5000 x g for ten minutes. This extraction procedure was repeated two additional times for each sample, resulting in a total extract volume of 2.25 mL. The supernatant was diluted to half the original concentration, to achieve a concentration of 8.34 ng/mL if 100% recovery was achieved. Three subsamples of extract from each sample were analyzed according to the Bt protein quantification procedure previously described.

Bt Cry3Bb1 Spike-and-Recovery

Bt Cry3Bb1 recovery was measured from freshly-spiked and aged soil samples. A 0.5-g sample of soil was spiked with 125 μ L of a 300 ng/mL Cry3Bb1 solution, which resulted in a soil concentration of 75 ng/g. The control treatment was spiked with 125 μ L of PBST. Samples were aged for 0.25, 24, 48, or 96 hours, and were maintained at 25°C in the dark. There were four replicates of the Bt Cry3Bb1 treatment and one replicate of the control treatment at each time point. At each time point, the corresponding replicates were removed from the study and were extracted with 1 mL of either biomimetic buffer [14] or a high-salt,

pH 10.5 buffer [15]. A description of these buffers can be found in Table 1. Samples were placed on an orbital shaker for one hour, and were centrifuged at 5000 x g for ten minutes. This extraction procedure was repeated two additional times for each sample, resulting in a total extract volume of 3 mL. Three subsamples of extract from each sample were analyzed according to the Bt Cry3Bb1 protein quantification procedure previously described.

Statistical Analysis

Percent recovery of Bt Cry1F from soil and sediment, as well as Bt Cry3Bb1 protein from soil was calculated for each buffer used at each time point in the study. Statistical Analysis System (SAS, v. 9.1) was used to compare mean percentage recovery of each protein for each matrix and buffer at each individual time point used using Tukey's Studentized Range test and Dunnett's t-test. Significance was determined at $p < 0.05$.

Short-Term Soil Monitoring Study

Study Description

A short-term monitoring study was conducted for Bt Cry1F protein. Soil for this study was collected from Bennett Farm, a 160-acre farm located in Story County, IA. A composite of soil collected from each of four field plots on Bennett Farm was analyzed by Midwest Laboratories, Inc. (Omaha, NE) using standard procedures. The soil was characterized as clay loam, with 32% sand, 39% silt, and 29% clay. Organic matter was 4.1% and soil pH was 7.4. Ten soil samples were collected from each of three field plots containing Pioneer Brand Seed Corn with Herculex® 1 (Pioneer; Johnston, IA), and one field plot that contained isoline corn and served as the conventional corn control. Soil was collected during anthesis (VT stage) in July 2006, after maturity (R6 stage) in September 2006, and following harvest in November 2006. In addition, leaf material was taken from corn plants in each field plot at each sampling time to determine the average Bt Cry1F concentration in corn leaves from all four plots. All soil samples were taken using a golf-cup cutter, which removed a soil core that was ~ 7 cm in diameter and 20 cm long. In order to determine where the greatest concentration of Bt Cry1F protein was in each field plot, four samples were taken between rows, three samples were taken within a row, and three samples were taken at the root zone of the plant in each of the four field plots sampled. These soil samples were randomly collected from between rows, within rows, and at the root zone in each of the field plots at each sampling time. Individual samples (n=10 per sampling time) were sieved using a 2.36-mm mesh sieve. Following sieving, the soil sample was visually inspected for plant material, which was removed by hand. A 5-g sample of sieved soil was weighed stored at -4°C until extraction and analysis.

Bt Cry1F Quantification

Bt Cry1F protein was extracted from each 5-g sieved soil sample using 10-mL biomimetic buffer [14] (Table 1). The soil was extracted on an orbital shaker at 300 rpm for one hour. Samples were centrifuged for 10 minutes at 3000 rpm and the supernatant was removed. This process was repeated two more times for each sample, resulting in a total supernatant volume of 30 mL. Bt Cry1F protein was extracted from Herculex 1® and isoline corn leaf at each of the collection times during the study. This was done by homogenizing three 20-mg samples of corn leaf collected from each field plot at each sampling time for one minute in 1-mL PBST with a Delta® 9” bench-top drill press (Delta Machinery, Jackson, TN, USA). Extracts were allowed to settle for ~15 min., and the supernatant was removed for analysis. Analysis was conducted using the previously described ELISA procedure for Bt Cry1F protein.

Western Blot and ESI-MS/MS Confirmation of Bt Protein Biological Activity

Study Description

A spike-and-recovery study that was previously conducted in this lab, determined the effects of the addition of 0, 0.5, 5, 10, or 50 mg/mL bovine pancreatic trypsin to a bicarbonate buffer on the extraction efficiency of Bt Cry1F from soil. Based on the results from this study (Figure 1, unpublished data), it appeared that the antibodies on the ELISA plate were able to bind cleaved protein fragments. This would result in a false increased recovery, because these cleaved fragments of Bt protein are not biologically active and would not confer toxicity to target or non-target organisms. Therefore, the present study was designed to determine whether low concentrations of trypsin (i.e., 0.5 or 5 mg/mL) were cleaving the protein or increasing the recovery of the intact protein from soil, using Western blot or electron spray ionization (ESI) – mass spectrometry (MS) as a chemical-confirmatory tool. In addition, this study was aimed at determining the usefulness of either Western blot or ESI-MS/MS as a method of confirming ELISA results, that is, determining whether extracts from environmental matrices contain biologically active intact Bt protein or cleaved Bt protein fragments.

Western Blot Procedure

A solution containing 10 ng/μL purified, *E. coli*-produced Bt Cry1F protein in PBST was treated with 0, 0.5, 5, or 10 mg/mL bovine pancreatic trypsin in high-salt, pH 10.5 buffer. Each treatment had only one replicate. Treated samples were mixed for one hour on an orbital shaker. The Western blot analysis was conducted in Iowa State University’s Department of Food Science and Human Nutrition. For the Western blot separation, the

treated samples were diluted with 2X reducing sample buffer [4 g sodium dodecyl sulfate (SDS), 25 mL Tris/SDS (6.05 g Tris base, 0.4 g SDS, 25 mL deionized water), 20 mL glycerol, 2 mL β -mercaptoanol, 1 mg bromophenol blue in 100 mL total deionized water]. A total of 1-10 ng Cry1F protein per lane was used for separation. Protein separation was carried out on a discontinuous (4% stacking, 10% resolving) SDS-polyacrylamide gel (PAGE; 30% acrylamide/bis solution; BioRad; Hercules, CA), which was followed by transfer to a polyvinylidene fluoride (PVDF) membrane (BioRad; Hercules, CA) at 800-100 volts for 3 hours. Blocking was done in 7% nonfat dry milk in tris-buffered saline with Tween-20® (TBST). The blot was incubated in 27.2 μ g (1:2000) rabbit polyclonal primary antibody overnight, followed by 3.2 μ g (1:1000) secondary antibody (goat; anti-rabbit; horse-radish peroxidase conjugated; Santa Cruz Biotechnology; Santa Cruz, CA) for one hour. Protein detection was done using the ECLPlus Detection System (Amersham Biosciences; Piscataway, NJ) and the Storm 840 Chemiluminescence Imager.

Electron Spray Ionization (ESI)-Mass Spectrometry (MS)/MS Procedure

A solution containing 200 pmol Bt Cry3Bb1 dissolved in PBST, was treated with 0, 0.5, 5, or 10 mg/mL bovine pancreatic trypsin. There was one replicate of each treatment. Each replicate was mixed on an orbital shaker for one hour prior to analysis. Analysis was conducted at the Iowa State University Plant Science Institute Proteomics Facility. The mass spectrometry was done according to standard procedures used by the Proteomics Facility. Electron-spray ionization (ESI) mass spectrometry was done using a Q-Star XL Hybrid LC/MS/MS Quadrupole-TOF mass spectrometer (Applied Biosystems; Foster City, CA). The Proteomics Facility is also able to identify proteins or peptides from chromatographic separations using Mascot software (Proteome Software, Inc; Portland, OR), if these proteins are available in a protein database. Mascot software was used in this study to identify the peaks represented by peptides cleaved from intact Cry3Bb1 protein.

Solid-Phase Extraction (SPE) of Bt Cry3Bb1 Protein

Study Design

The following 3-mL solid phase extraction (SPE) tubes were used for SPE of purified, *E. coli*-produced Bt Cry3Bb1 protein: Supelclean® SPE tubes (SUPELCO; Bellefonte, PA) containing weak-cation (WCX), weak-anion (WAX), strong-cation (SCX), or strong-anion exchange (SAX) packings; Supelclean® (SUPELCO; Bellefonte, PA) SPE tubes containing C-4 or C-18 (ENVI-18®) packings; or Oasis® HLB SPE tube (Waters; Milford, MA) containing C-18 packing. A 60-mL solution of 10 ng/mL Bt Cry3Bb1 dissolved in biomimetic buffer was loaded onto a conditioned column. There were at least two replicates

of each SPE tube type per procedure attempted, and there were five procedures attempted total (Table 2). The following general procedure was used for all five procedures, and closely followed the manufacturer's instructions: each tube was conditioned to activate the packing material, the sample was loaded and slowly passed through the tube, the packing was washed to remove any unwanted interference compounds, and the packing was rinsed to elute the compound of interest (e.g. Bt Cry3Bb1). At each step in each procedure utilized, except the conditioning step, solution was collected as it eluted off each SPE tube. This solution was stored at -4°C until analysis by ELISA using the previously described method for Bt Cry3Bb1.

Statistical Analysis

Percent retention of Bt Cry3Bb1 protein loaded on the SPE tube following sample loading and interference elution steps was calculated for each of the protocols and types of SPE tubes listed in Table 2. In addition, percentage elution of Cry3Bb1 loaded on the SPE tube following the analyte elution step was calculated for each protocol attempted and each type of SPE tube used. SAS (v. 9.1) was used to compare mean percentage retention and elution for each SPE protocol and each type of SPE tube used with Tukey's Studentized Range test and Dunnett's t-test. Significance was defined at $p < 0.05$.

Results

Spike-and-Recovery of Bt Cry1F and Bt Cry3Bb1

There was no Bt Cry1F or Bt Cry3Bb1 protein measured in any of the extracts from the PBST-spiked soil or sediment at any time point in the study. Bt Cry1F recovery from soil was highest for the 0.25-hr. extracts, and ranged from 70-27% depending on the buffer used (Figure 2). Significantly higher recovery was achieved using biomimetic buffer with and without the addition of 0.5 mg/mL bovine pancreatic trypsin at all time points except 48-hr. The addition of trypsin to the biomimetic buffer increased the extraction efficiency of the buffer, at the 24- and 48-hr. time points but not at the 0.25- or 96-hour time points. The addition of 0.5 mg/mL trypsin to the high-salt, pH 10.5 buffer increased the recovery of Bt Cry1F at the 0.25- and 24-hr. time points (Figure 2). Bt Cry1F recovery from sediment was again highest at the 0.25-hr. time point, ranging from 72-34% depending on the buffer used (Figure 3). Overall, Bt Cry1F extraction efficiency from sediment was much better than that achieved from soil. There was a trend of increased recovery of Cry1F with biomimetic buffer, with and without trypsin. This increased recovery was higher when compared to the high-salt, pH 10.5 buffer with trypsin at the 24-, 48-, and 96-hr. time points. The biomimetic

buffer, with and without trypsin, and the high-salt, pH 10.5 buffer produced similar recoveries of Bt Cry1F from sediment at the 24-, 48-, and 96-hr. time points. At the 0.25-hr. time-point biomimetic buffer with trypsin added had a significantly higher recovery of Bt Cry1F than the other three buffers used (Figure 3). There was no recovery of Bt Cry3Bb1 from soil at any time point using the biomimetic buffer. The high-salt, pH 10.5 buffer had the greatest recovery of Bt Cry3Bb1 protein at the 0.25-hr. time point, with 41%. Cry3Bb1 recovery decreased to 15% at the 96-hr. time point (Figure 4).

Short-Term Soil Monitoring Study

There was no Bt Cry1F measured in any of the corn leaf samples taken from the conventional corn control plot at any time during the study. In addition, there was no Cry1F measured in any of the soil extracts collected from the control plot at any time during the study. The concentration of Cry1F in the corn leaves collected from each of the three Herculex 1® field plots was very similar at anthesis and senescence, and ranged from 12.3-17.1 µg/g (Table 3). The post-harvest corn leaf samples likely contained corn residue from the entire field in which the study was taking place due to mixing from the harvesting equipment. However, Bt Cry1F was measured in two of the nine leaf samples. The remaining leaf samples did not contain Cry1F (Table 3). There was no Bt Cry1F measured in any of the soil samples taken from any of the three Herculex 1® field plots at any time point during the study, regardless of how close to the corn plant the samples were taken.

Bt Protein Analysis using Western Blot and ESI-MS/MS

The Western blot analysis indicated a Bt Cry1F protein standard band in lane 2 of 65 kD molecular weight, which is the weight of the purified, *E. coli*-produced Cry1F protein (Figure 5). The purified Bt Cry1F standard contained an impurity with a mass of approximately 41 kD, and having similar structure to Cry1F. Protein cleavage is indicated in the lanes 4 and 5, containing Cry1F protein with 5 and 10 mg/mL trypsin. The cleaved peptides are less than 30 kD in mass. Protein cleavage may also be indicated in lane 3, containing Cry1F protein with 0.5 mg/mL trypsin, however, clear visualization could not be achieved using Western blot (Figure 5).

ESI-MS/MS did not achieve any result. While a spectrum for Bt Cry3Bb1 protein was achieved, any peptides that may have resulted from Cry3Bb1 cleavage due to the addition of trypsin could not be identified.

SPE of Bt Cry3Bb1 Protein

Overall, retention of Bt Cry3Bb1 to the SPE tubes used in each of the five protocols was good and ranged from 65-100% of Bt Cry3Bb1 loaded following both the sample loading and interference washing steps (Figure 6). There was no elution of Bt Cry3Bb1 analyte from the SPE tubes in any of the protocols used, therefore, the protein that was retained on the column was tightly adsorbed to the packing material and could not be eluted. Protocol 3, which used SAX SPE tubes, had the highest retention of all five protocols used with 100% retention. Protocol 2 had statistically lower retention of Bt Cry3Bb1 protein to the HLB, SCX, and WCX tubes used when compared to the other four protocols that resulted in similar retentions (Figure 6). This is likely due to the use of the HLB SPE tube in this protocol as this type of SPE tube had 70% retention, while the SCX and WCX tubes had a 81% and 76% retention, respectively. There was no statistical difference between any of the SPE tubes used in all five protocols, however, the HLB and NH₂ SPE tubes had the lowest retention with 70% and 66%, respectively. The ENVI-18 and the SAX SPE tubes had the best overall retention with 95% and 94%, respectively.

Discussion

Previous studies in this laboratory (Figure 1, unpublished data) have shown a markedly increased recovery of Bt Cry1F protein from soil with the addition of bovine pancreatic trypsin to a bicarbonate extraction buffer. The present study did not repeat the results seen at the 0.5 mg/mL level; however, different extraction buffers were used in this case. In addition, Western blot results indicate that the addition of 5 and 10 mg/mL trypsin to purified Cry1F protein produces cleaved peptides with a molecular weight of less than 30 kD. It appears that the addition of 0.5 mg/mL trypsin also results in cleavage of Cry1F. However, Western blot analysis should be repeated in order to confirm this result. Therefore, the use of proteases to increase the extraction efficiency of Bt proteins from environmental matrices may not be a viable option. Of the two buffers that have been published for the extraction of Bt proteins from soil, the biomimetic buffer resulted in higher recoveries of Bt Cry1F protein from freshly-spiked soil and sediment. This increased extraction efficiency became less pronounced for the 24-, 48-, and 96-hour time points. The present study represents the first attempt to develop a method for the extraction and quantification Bt proteins from sediment. A 72% recovery of Bt Cry1F from freshly-spiked sediment was achieved using the biomimetic buffer, however, 96-hours after spiking recovery was less than 20%. Overcoming the adsorption of Bt proteins to surface-active particles (e.g. clay, humic acids)

in soil and sediment is a challenge that has yet to be resolved. In the present study, a 70% recovery of Bt Cry1F from freshly-spiked soil was achieved, but less than 10% was recovered using the same method 96-hours after spiking. A similar trend was seen with Bt Cry3Bb1 recovery from soil, with a 41% recovery from freshly-spiked soil and only a 15% recovery after 96-hours. This study is also the first to report a new-found challenge in the analysis of Bt proteins from environmental matrices, which is the differential recovery of Bt Cry1F and Bt Cry3Bb1 proteins. Biomimetic buffer resulted in the highest recovery of Bt Cry1F from soil and sediment at all time points in the spike-and-recovery study. However, no recovery was achieved using biomimetic buffer and the same extraction method in soil spiked with Bt Cry3Bb1 at any time point. The high-salt, pH 10.5 buffer is the best extraction buffer found thus far for extracting Bt Cry3Bb1 protein from soil, however, the highest recovery achieved was 41%. It is yet to be determined if this differential recovery exists for all Cry1 and Cry3 proteins, but this could represent a difficulty in investigating the environmental fate of stacked-trait crops expressing both Cry1Ab and Cry3Bb1 proteins, such as YieldGard Plus® corn.

The decrease in Bt Cry1F recovery from soil 24-, 48-, and 96-hours after spiking in the spike-and-recovery study, may explain why there was no Cry1F measured in the extracts of soil samples taken from three field plots containing Herculex 1® corn in the short-term monitoring study. In addition, the clay loam soil from Bennett Farm had 29% clay content, which is higher than the sandy loam soil used in the spike-and-recovery study. The increased clay content provides more surface area for Bt proteins to adsorb to, which would make extraction difficult. It is also possible that any Bt protein that entered the soil via pollen shed, sloughing of root cells, or root exudates degraded quickly leaving no Cry1F to quantify. Bt Cry1F has been found to degrade rapidly in soil, with a reported half-life in one study of less than one day [7].

A suitable solid-phase extraction method for Bt proteins did not result from this study. However, good retention to the SPE tubes following sample loading and interference elution was achieved. It appears that Bt Cry3Bb1 protein adsorbed tightly to the SPE packing, hence the high retention, but a method to elute the protein was not found in the present study. ENVI-18 and SAX SPE tubes had the best retention for all protocols used, and may be promising candidates for future SPE method development. Overall, the highest retention was found using protocol 3 and SAX SPE tubes. For this method the pH of the extract containing Cry3Bb1 was adjusted to pH 9. This method may be ideal for Bt Cry3Bb1, for which the high-salt, pH 10.5 buffer had the largest extraction efficiency from soil.

Acknowledgement

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Table 1. Description of the extraction buffers utilized for recovery of Bt Cry1F and Cry3Bb1 from environmental matrices.

Buffer	Chemical	Concentration
Biomimetic (Shan et al. 2005)	NaCl	0.36 M
	Na ₂ SO ₄	24 mM
	KCl	8 mM
	NaHCO ₃	20 mM
	CaCl ₂	12 mM
	MgCl ₂	98 mM
	Sodium Taurocholate	0.7% (w/v)
	Bovine Serum Albumin (BSA)	0.5% (w/v)
	pH	7.5
High-salt/High-pH (Palm et al. 1996)	B ₄ Na ₂ O ₇	50 mM
	KCl	0.75 M
	L-Ascorbic acid	10 mM
	Tween-20®	0.075% (v/v)
	pH	10.5

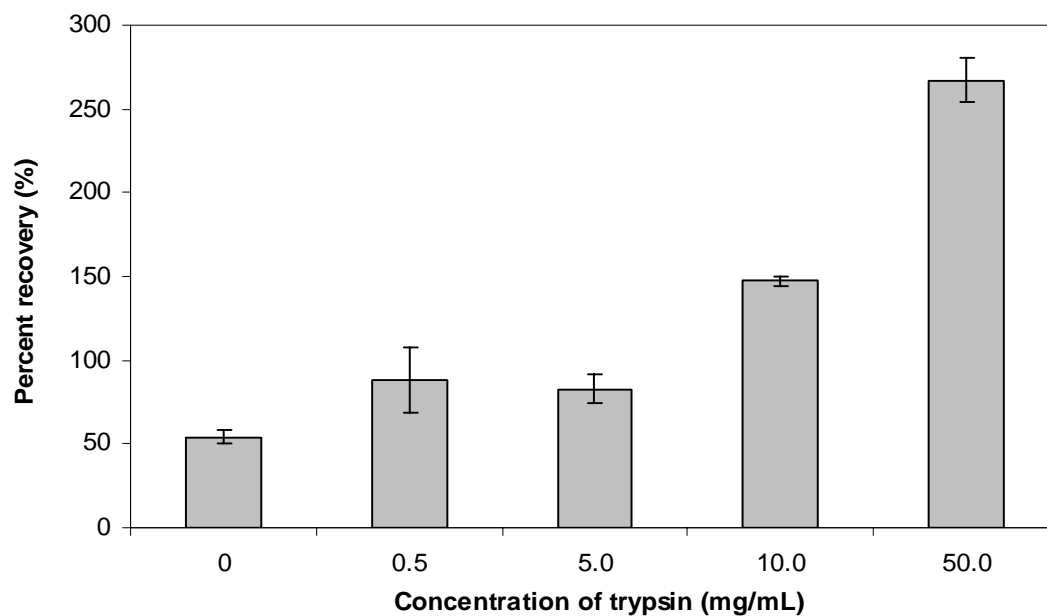


Figure 1. Recovery of Cry1F protein, measured by enzyme-linked immunosorbent assay (ELISA), from soil using bicarbonate buffer with several concentrations of trypsin.

Table 2. Summary of procedures used to develop solid-phase extraction (SPE) methodologies for transgenic Bt proteins.

Protocol No.	SPE Tubes	Sample Pretreatment	Conditioning Solvents	Interference Elution Solvents	Analyte Elution Solvent
1	WCX, SCX	None	Methanol, DI water, PBST (5 mL)	PBST (5 mL)	0.2 M CaCl ₂ Buffer (4 mL)
2	HLB, WCX, SCX	Adjust pH to 5.0	Methanol, DI water (3 mL)	5% Methanol; 2% ammonium (4 mL)	2% Glacial acetic acid (4 mL)
3	SAX	Adjust pH to 9.0	Methanol, DI water, pH 9.0 biomimetic buffer (3 mL)	5% Methanol (4 mL)	1% Glacial acetic acid (4 mL)
4	SAX, NH ₂ , ENVI-18	Adjust pH to 5.0	Methanol, DI water, pH 5.0 biomimetic buffer (3 mL)	pH 5.0 Biomimetic buffer (4 mL)	1% Glacial acetic acid (4 mL)
5	C-4	0.5% Trifluoroacetic acid (TFA, 300 µL)	50% Methanol, 0.1% TFA (1 mL)	0.1% TFA (1 mL)	75% Methanol/0.1% TFA (2 mL)

List of abbreviations used in Table 2: weak-cation exchange (WCX), strong-cation exchange (SCX), 18-carbon (HLB, ENVI-18), strong-anion exchange (SAX), amido acid (NH₂), 4-carbon (C-4), distilled (DI), phosphate-buffered saline with Tween-20® (PBST), calcium chloride (CaCl₂).

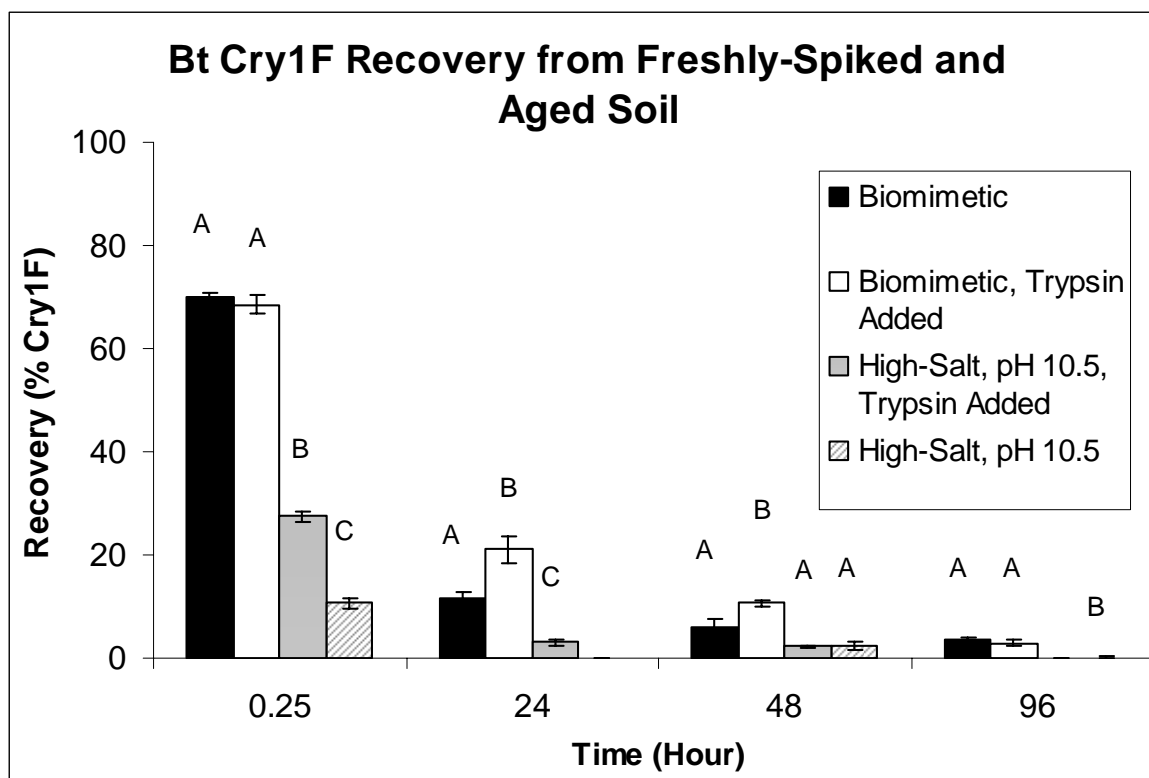


Figure 2. Average recovery (\pm SE) of Bt Cry1F from soil using four different extraction buffers. Bt Cry1F protein was quantified by enzyme-linked immunosorbent assay (ELISA). N = 2-8 replicates per buffer per time point. Means with no common letter differ significantly ($\alpha < 0.05$) at that time point.

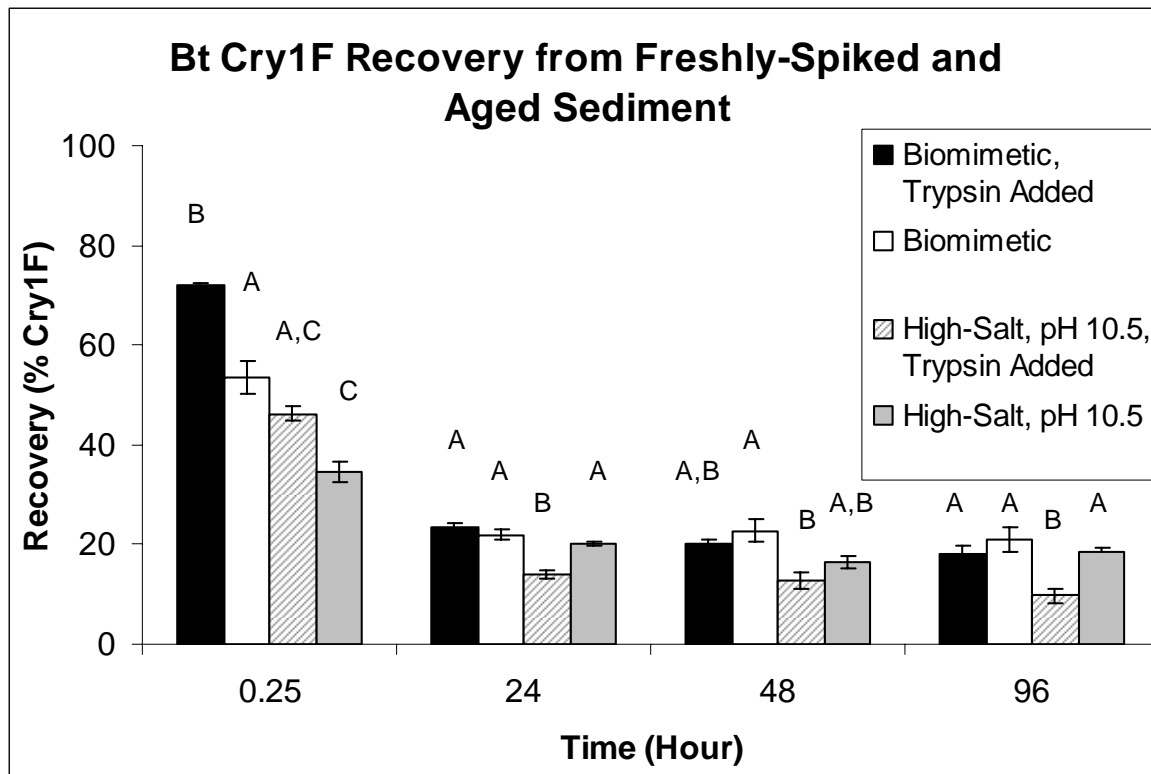


Figure 3. Average recovery (\pm SE) of Bt Cry1F from sediment using four different extraction buffers. Bt Cry1F protein was quantified using enzyme-linked immunosorbent assay (ELISA). N = 3-10 replicates per buffer per time point. Means with no common letter differ significantly ($\alpha < 0.05$) at that time point.

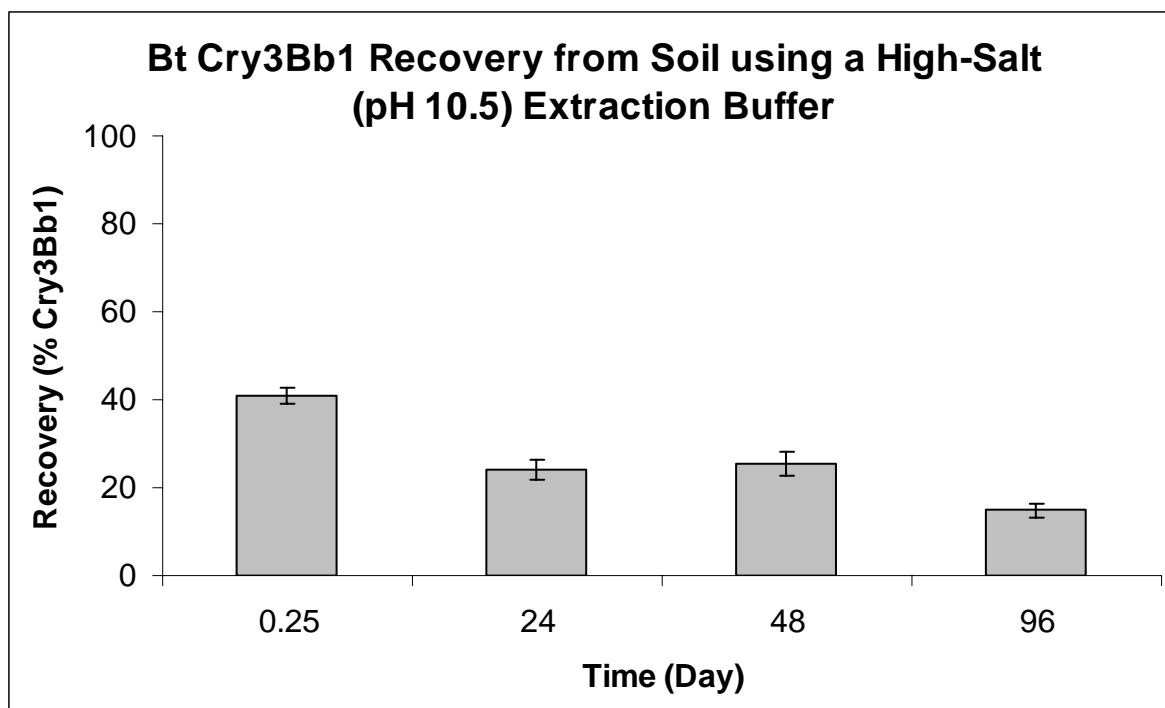


Figure 4. Average recovery (\pm SE) of Bt Cry3Bb1 from soil using a high-salt, high-pH (10.5) buffer. Bt Cry3Bb1 protein was quantified using enzyme-linked immunosorbent assay. N = 4 replicates per time point.

Table 3. Average (\pm SE) Bt Cry1F protein measured by enzyme-linked immunosorbent assay (ELISA) in corn leaf samples taken from a control plot and three Herculex 1® field plots during a short-term monitoring study. N = 1-3 corn leaves per field plot per sampling date.

Sampling Date	Life Stage	Plot	Average Bt Cry1F Conc. ($\mu\text{g/g} \pm \text{SE}$)
28-Jul-06	Anthesis	Control	0
28-Jul-06	Anthesis	1	15.5
28-Jul-06	Anthesis	2	17.1
28-Jul-06	Anthesis	3	16.6
26-Sep-06	Senescence	Control	0
26-Sep-06	Senescence	1	14.7 ± 1.4
26-Sep-06	Senescence	2	12.3 ± 3.4
26-Sep-06	Senescence	3	13.4 ± 1.6
16-Nov-06	Post-Harvest	Control	0
16-Nov-06	Post-Harvest	1	0.05 ± 0.05
16-Nov-06	Post-Harvest	2	0.03 ± 0.03
16-Nov-06	Post-Harvest	3	0

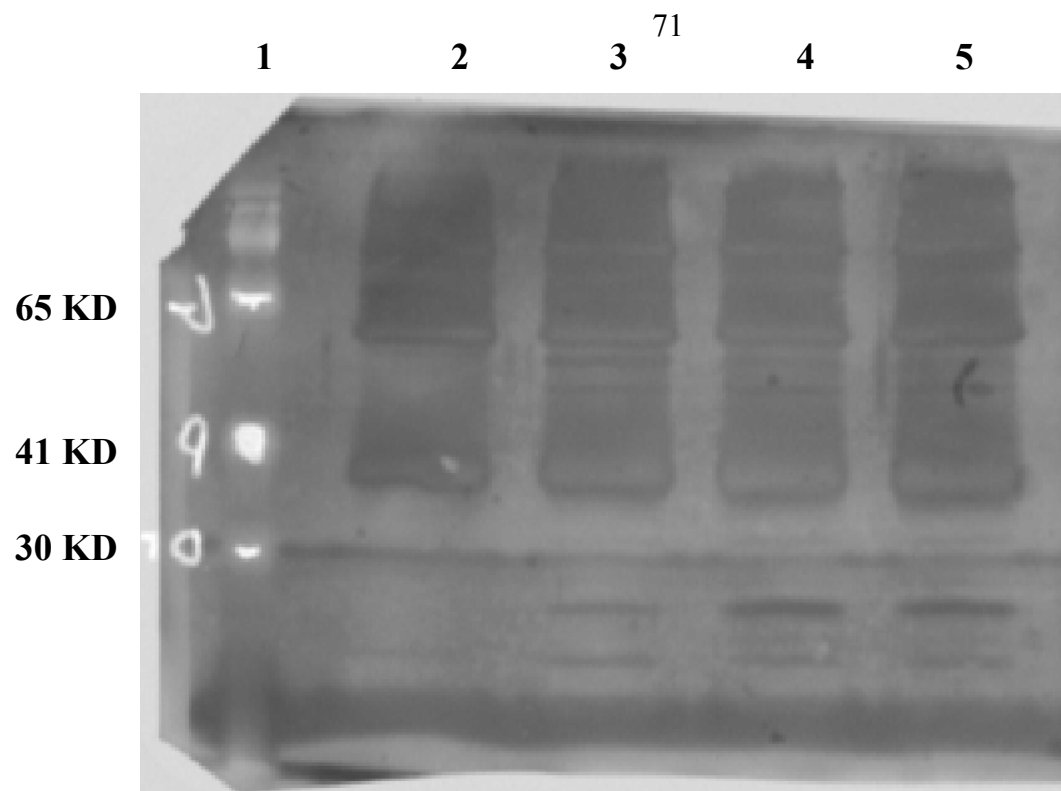


Figure 5. Western blot analysis of Bt Cry1F protein with the addition of 0, 0.5, 5, or 10 mg/mL bovine pancreatic trypsin. Lane 1 is the molecular weight marker containing 65, 41, and 30 KD standards. Lane 2 is purified Cry1F without trypsin. Lanes 3-5 are Cry1F protein with 0.5, 5, and 10 mg/mL trypsin, respectively.

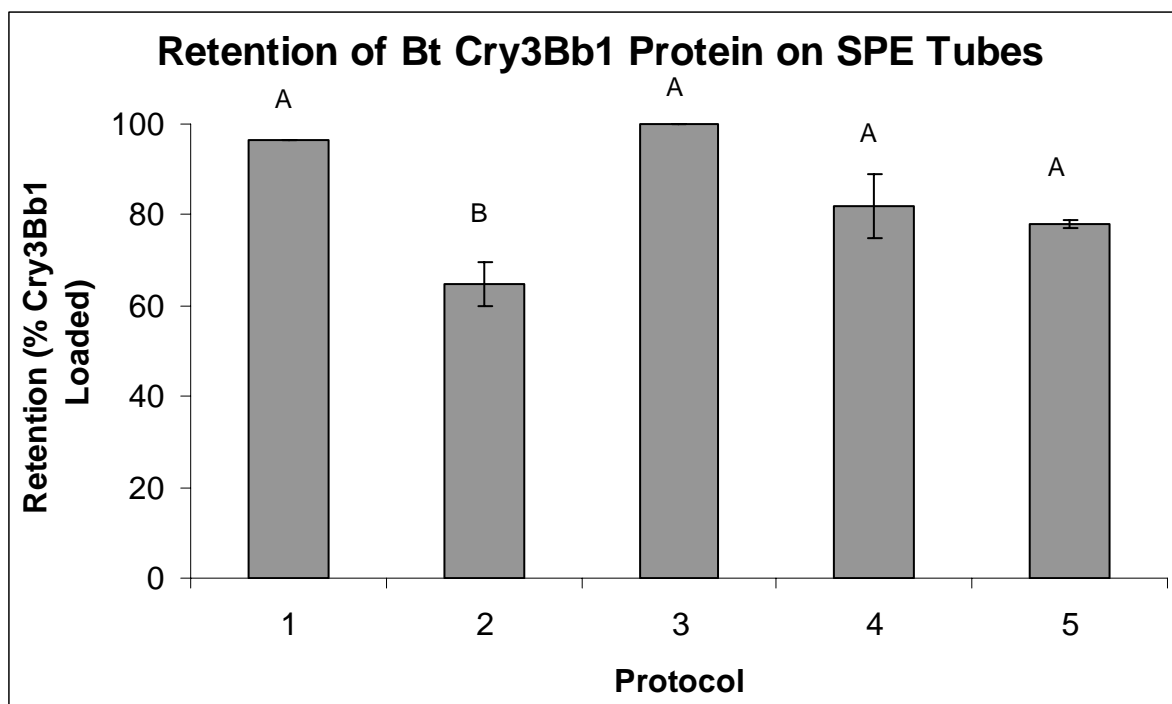


Figure 6. Average (\pm SE) retention of Bt Cry3Bb1 protein on solid-phase extraction (SPE) tubes, following sample loading and interference elution, using five different methods. N = 3-7 SPE tubes for each protocol.

CHAPTER 5. GENERAL CONCLUSIONS

In 2006, 19 million hectare (ha) of Bt crops were planted worldwide, which represented an 18 million ha increase since their introduction to the agricultural market in 1996 [1]. This trend of increasing worldwide acreage of Bt crops is likely to continue due to the addition of new Bt crops to the agricultural market, a larger numbers of target pest species protected against, and a decreased need for traditional chemical pesticides. At the same time, there will likely be an increase in the number of transgenic crops (e.g., pharma-crops) available, as the field of biotechnology expands. Therefore, it is important to establish methods to examine the environmental fate and non-target effects, both terrestrial and aquatic, of Bt proteins. Additionally, analytical methods that accurately and efficiently quantify Bt proteins from environmental matrices are currently lacking. In order to establish the ecological risk of these novel insecticides, reliable laboratory and field exposure and effects data are needed, and the sustainability of this new direction in agriculture must be established. This research was aimed at developing realistic laboratory methods to determine the terrestrial and aquatic fate of Bt proteins from transgenic corn, as well as to examine their potential aquatic non-target effects. In addition, several methodologies to improve the analysis of Bt proteins from environmental matrices were researched.

In the second chapter, methods were successfully developed to examine the aquatic fate of Bt proteins in decomposing crop residue and to determine the acute effects of Bt proteins on benthic aquatic invertebrates. Bt Cry3Bb1 was found to degrade exponentially, according to first-order kinetics, in aquatic microcosms with and without sediment. A short half-life of less than 3 days for Bt Cry3Bb1 protein was found for MON 863 corn leaf, stalk, and root residue. Therefore, Bt Cry3Bb1 will probably not be persistent in water, and exposure to non-target aquatic organisms will be primarily acute. The current extraction technique available for Bt Cry3Bb1 yields a relatively low recovery (less than 41%); therefore, the dissipation of Cry3Bb1 in the water column and the sediment could not be measured definitively. However, there is evidence from previous aquatic fate studies conducted in this laboratory, that Bt proteins adsorb to sediment and may persist in this environment due to decreased availability for microbial degradation. In order to further examine this possibility, more research needs to be conducted on extraction of Bt proteins from environmental matrices. MON 863 root extracts containing Bt Cry3Bb1 protein were found to have significant acute effects on the survival of *Chironomus dilutus* larvae in a ten-day static, partial-renewal study in the second chapter, at measured concentrations of 30 ng/mL and above. No effects on growth were found in this study. It is not known if these effects will be

seen in chironomid populations in the field, and more research is needed to determine the hazard of acute Bt protein exposure to aquatic invertebrates. The use of non-traditional aquatic toxicology models, such as the water scavenger beetle (Hydrophilidae) or predaceous diving beetle (Dytiscidae), may be an interesting model for future studies on the non-target effects of the coleopteran-active Bt Cry3Bb1 protein.

A similar short half-life of less than 6 days was found for Bt Cry3Bb1 protein in decomposing MON 863 corn leaf, stalk, and root in terrestrial fate studies in the third chapter. There was a trend seen in laboratory microcosms that contained macrodecomposers of longer half-life for Cry3Bb1 in MON 863 corn tissue, however, this trend was only statistically significant in the case of Cry3Bb1 in leaf residue. In addition, the difference in length of half-life seen in treatments with and without macrodecomposers, is not likely to be ecologically or toxicologically significant due to the acute nature of exposure in both treatments. Thus far, acute effects of Bt protein on non-target terrestrial organisms have not been found in the field. Bt Cry3Bb1 protein was qualitatively measured in the soil of laboratory microcosms in the third chapter. This indicates that the protein leaches out of decomposing crop residue and adsorbs to soil. The dissipation of Cry3Bb1 in the soil could not be studied due to the low extraction efficiency of this protein using the best-available current analytical method. More research is needed on improving the analytical method of this protein before its dissipation in soil and sediment can be reliably investigated.

The fourth chapter included research aimed at improving the current analytical method for Bt proteins. This chapter represents the first known study to report an analytical method for any Bt protein from sediment. In addition, it is also the first time that the differential recovery of Bt Cry1F and Bt Cry3Bb1 protein from soil has been reported. More research is needed to determine if this is the case for all Cry1 and Cry3 proteins, however, this differential recovery could pose an even greater analytical challenge when studying the environmental fate of stacked-trait Bt crops. In addition, results from the fourth chapter confirmed a flaw in the use of enzyme-linked immunosorbent assay (ELISA) for quantification of Bt proteins because Western blot analysis confirmed that ELISA quantifies peptides cleaved from Bt proteins, as well as the intact and biologically active protein. This discovery reveals a need for a method of chemical confirmation coupled to ELISA quantification of Bt proteins because soil and sediment extracts may contain both cleaved and intact protein. Although electron-spray ionization coupled with mass spectrometry (ESI-MS/MS) did not yield a successful method of Bt protein analysis in the fourth chapter, it is a promising tool and future research on ESI-MS/MS and liquid chromatography methods of Bt protein analysis should be conducted. Using either of these two methods, solid-phase

extraction (SPE) will be needed to clean-up and concentrate extracts from environmental matrices. In the fourth chapter, strong-anion exchange (SAX) and C-18 SPE tubes resulted in a high percentage of Bt Cry3Bb1 retention after sample loading and interference elution, however, no method was established that resulted in Cry3Bb1 elution from the SPE packing material.

References

1. James C. 2006. Global status of commercialized biotech/GM crops: 2006. *ISAAA Briefs* No. 35. ISAAA: Ithaca, NY.